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THE UNIVERSITY OF ALBERTA

INTESTINAL MORPHOLOGY AND BRUSH BORDER MEMBRANE ADAPTATION IN HEALTH AND DISEASE

BY

Ⓒ MONIKA KEELAN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

OF MASTER OF SCIENCE

IN

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EDMONTON, ALBERTA

FALL, 1986

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THE UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Intestinal Morphology and Brush Border Membrane Adaptation in Health and Disease submitted by Monika Keelan in partial fulfilment of the requirements for the degree of Master of Science in Experimental Medicine.

Supervisor

M. T. Clandinin

Date: June 19, 1986

To

My parents and teachers
who taught me so much, yet made me
realize how much more there is to learn.

My husband, David
for his love and understanding during
my pursuit of education.

My twin sons, Matthew and Sean
in loving memory.

ABSTRACT

Aging, diabetes mellitus, chronic ethanol consumption and external abdominal irradiation have been associated with alterations in intestinal transport function in animal models. The mechanism of this "adaptation" in transport function has not been defined. It is suggested that changes in brush border membrane (BBM) composition and morphology of the intestine may provide a possible mechanism whereby changes in transport function could be mediated. This research proposal examined the marker enzyme activity and lipid composition of the BBM as well as intestinal morphology to study their possible relationship with transport function. BBM enzyme marker activity and intestinal villus morphology represented part of the biochemical and structural adaptation in each of the four animal models, yet the changes observed were not related to changes in transport function. Changes in BBM lipid composition, in particular phospholipid composition, were observed with every animal model studied. The type of phospholipids in the BBM alter physical and chemical properties of the membrane which, in turn, may alter permeability properties of the BBM as well as activity of carrier proteins involved in active transport. These studies support the hypothesis that changes in BBM phospholipids represent one of the mechanisms associated with the adaptation of intestinal transport function.

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CHAPTER I.

INTRODUCTION

I.1 GENERAL

Adaptation is described in Webster's dictionary as the "modification of an organism or its parts that makes it more fit for existence under the conditions of its environment". The concept of intestinal adaptation originated with the the observation of the intestine's amazing ability to adapt following resection. Following proximal resection, cell proliferation is stimulated in the distal intestine to such an extent that the distal intestinal mucosa resembles that of the now missing proximal intestine. Many states in health and disease have been associated with a functional, structural and biochemical adaptive response in the intestine. Several functional changes have been described. With aging, cholesterol uptake is decreased in older rabbits (Thomson, 1981a), but increased in older rats (Hollander and Morgan, 1979). In diabetic rats, the passive uptake of cholesterol and fatty acids (Thomson 1980, 1983) and the active uptake of glucose (Thomson, 1981b) are increased. Fasting creates no increase in cholesterol uptake in diabetic rats (Thomson, 1981b). Cholesterol and fatty acid uptake are decreased following chronic ethanol consumption in rabbits (Thomson, 1984a). Following exposure to external abdominal irradiation, active and passive uptake of nutrients is decreased even up to 14 days (Thomson et al, 1983,1984a,1984b).

What factors contribute to these changes in intestinal function that lead to intestinal adaptation? To be taken up from the lumenal bulk phase, nutrients must cross two barriers in series: the unstirred water layer and the brush border membrane. The rate of nutrient uptake into the enterocyte is determined by the characteristics and dimensions of these two barriers (Dietschy, 1973; Dietschy et al, 1971; Dietschy and Westergaard, 1975; Thomson and Dietschy, 1977; Westergaard and Dietschy, 1974; Wilson and Dietschy, 1975; Winne, 1976).

Effective resistance of the unstirred water layer (UWL) is determined by its thickness and surface area, but also by the probe molecule's free diffusion coefficient (Westergaard and Dietschy, 1974). Passively absorbed solute uptake (fatty acids, fatty alcohols, cholesterol) is rate-limited by the UWL (Westergaard and Dietschy, 1974). UWL thickness is reported at approximately 100 - 500 m in rodents (Westergaard and Dietschy, 1974; Wilson and Dietschy, 1975; Winne, 1976) and greater than 500 m in man (Read et al, 1976; 1977). UWL surface area is reported at less than 1% of the underlying membrane in rabbits (Westergaard and Dietschy, 1974; Wilson and Dietschy, 1975). Studies of intestinal transport function suggest that changes in UWL may not always explain the direction and magnitude of observed nutrient transport.

Although the dimensions of the brush border membrane mucosal surface area have been reported by many workers, the reports are not consistent with one another (Kapadia and Baker, 1976; Boyne et al, 1966; Penzes and Skala, 1977; Clarke, 1972). An accurate measure of villus and mucosal surface areas is important to understand the impact of structural changes in intestinal morphology on nutrient absorption.

The brush border membrane (BBM) is the first membrane nutrients must pass to gain entry into the body. The biochemical properties of this membrane may therefore be very important for regulating nutrient traffic into the body. Membrane lipids are of particular interest because they may influence the passive permeability properties of the BBM and, therefore intestinal nutrient transport.

Understanding the mechanisms of intestinal adaptation (functional, structural or biochemical) will aid in the identification of signals which mediate the adaptive response.

I.2 BRUSH BORDER MEMBRANE

Structure

Cellular membranes are involved in many vital cell functions. The plasma membrane of the enterocyte is no exception. The brush border membrane (BBM) is a striated border on the enterocyte (Figure I-1) which separates the enterocyte from the lumen (compartmentalization), acts as a permeability barrier, is the site of active transport into the cell, is involved in endocytosis (macrotransport), is the site of enzymes, receptors and antigens, and maintains cell shape and motility via the cytoskeleton and actin-myosin filaments, respectively.

Singer and Nicholson (1972) developed the fluid mosaic model of membrane structure through the use of sophisticated techniques such as X-ray diffraction, fluorescence, spin labelling, differential scanning calorimetry, nuclear-magnetic resonance, circular dichroism, and optical rotary dispersion. This model describes a continuous lipid bilayer throughout which protein molecules are embedded (Figure I-2). In addition, the lipids and proteins are capable of dynamic movement. Two conclusions were derived from this model: 1) the lipids and proteins in the membrane must be arranged in a tightly packed water-excluding mosaic. This would allow the apolar hydrophobic regions of proteins and lipids to interact in the core of the membrane, while the hydrophilic regions are solvated in an aqueous environment; 2) The individual components move freely within the plane of the membrane: rotational (axial) movement and lateral (translational) movement. Weak, non-covalent interactions occur between the apolar residues due to the synchronous oscillation of their electronic clouds (van der Waal's forces), but more important in the indirect energetic advantage of excluding water (hydrophobic force). When summed over many hydrophobic residues, the hydrophobic forces provide a powerful deterrent to disaggregation.

Composition

The BBM is composed of lipids (60%), protein (40%), and carbohydrate (<10%). The lipids are divided into two categories: 1) bulk phase and 2) annular or boundary proteins, both categories having the same composition. The lipids provide a structural backbone in which proteins are embedded. The carbohydrate is found primarily on the external surface, covalently attached to proteins and lipids, and is known as the glycocalyx. The presence of many sialic acid residues on the glycocalyx are responsible for imparting a net negative charge to the enterocyte. Water is also associated to the membrane (20% of mass) as well as many chelated Ca^{2+} and Mg^{2+} ions.

a) Lipids

Few studies have reported the lipid composition of the brush border membrane (Hauser et al, 1980; Forstner et al, 1968; Millington & Gritchley, 1968; Billington & Nayudu, 1978; Christiansen & Carlsen, 1981). Three lipid classes make up the BBM: phospholipids, neutral lipids and glycolipids in a ratio of 1:1:2 (Forstner et al, 1968). The lipids are distributed asymmetrically across the bilayer due to structural constraints of the molecules. Phospholipids may be broken down into three categories:

i) choline phospholipids - lysolecithin, lecithin, and sphinogomyelin. These less reactive phospholipids are more frequently located on the outer layer of the membrane bilayer.

ii) amine phospholipids - phosphatidyl serine, lysophosphatidyl ethanolamine, and phosphatidyl ethanolamine. These more reactive phospholipids are more frequently located on the inner layer of the membrane bilayer.

iii) other phospholipids - phosphatidyl inositol and phosphatidic acid. These phospholipids also exhibit a preference for the inner membrane layer.

Phospholipids are amphipathic in nature. They are composed of a charged polar head group and 1-2 nonpolar aliphatic hydrocarbon chains. The hydrophobic moiety are the fatty acids which are esterified via the glycerol backbone and the phosphate ester to the hydrophilic base. These fatty acids are generally 14-24 carbons in length. The most abundant fatty acids are the 16 and 18 carbon series.

Neutral lipids in the BBM consist primarily of cholesterol, but lesser amounts of diglyceride, triglyceride, free fatty acid, and cholesterol ester are also present.

Glycolipids present in the BBM include cerebrosides, ceramide, ceramide monohexoside, ceramide dihexoside, ceramide trihexoside, ceramide tetrahexoside, ceramide sulphatide, and gangliosides (predominantly GM_3).

b) Proteins

The protein components of mouse duodenal brush border membranes have been analyzed (Billington & Nayudu, 1975). Many workers have described the enzymes associated with the brush border membrane (Hartmann et al, 1982; Nordstrom & Dahlqvist, 1972).

There are two classes of proteins:

i) intrinsic or integral proteins:

These proteins are embedded within the lipid bilayer and comprise approximately 70% of the total membrane protein. Alkaline phosphatase and maltase are examples of intrinsic membrane proteins of the BBM.

ii) extrinsic or peripheral proteins

These proteins are loosely associated with the membrane surface via weak, non-covalent interactions. Sucrase is an extrinsic BBM protein, and part of the sucrase-isomaltase complex. The cytoskeleton proteins are also extrinsic to the BBM. Proteins are asymmetrically localized in the membrane in order to orient the function of the protein to a specific side of the membrane.

Asymmetric Distribution

Lipids are synthesized and inserted on the cytoplasmic side of the membrane only. Asymmetry is achieved through "flip-flop" to the external side of the membrane.

Proteins are inserted into the membrane during biosynthesis, or synthesized free in the cytosol. Proteolysis fixes the orientation of the membrane protein. Glycosylation may asymmetrically localize the enzyme on the outside of the membrane.

Physicochemical Properties

Phospholipids and cholesterol are the membrane components which determine the physicochemical properties. At physiological temperatures, the bulk of the lipids in biological membranes are in the liquid-crystalline state. Biological membrane must be in the liquid-crystalline state to maintain membrane functions. The liquid-crystalline state is influenced by:

1) The length and degree of unsaturation of the phospholipid fatty acyl chains. Long unsaturated fatty acyl chains (i.e. 20:4) are more fluid than short saturated fatty acyl chains (i.e. 16:0).
2) The nature of the phospholipid polar head group influences the mobility of the fatty acyl chains. A large polar head group (e.g. phosphatidyl choline) allows more mobility than a small head group (e.g. phosphatidyl ethanolamine).
3) The amount of cholesterol influences the motional freedom of the fatty acyl chains. Cholesterol is a rigid planar molecule which decreases the motional freedom of nearby fatty acyl chains. Cholesterol increases the electrostatic interaction between the hydroxyl and phosphate groups which help maintain order and rigidity in the bilayer. Cholesterol functions to decrease the fluidity of a membrane to a state intermediate between gel and liquid-crystalline.

4) The temperature influences molecular packing and motional freedom of the fatty acyl chains. At low temperatures, the membrane is in the gel state, which describes the close molecular packing of fatty acyl chains in an *all-trans* state. With an increase in temperature, fatty acyl chains change to a fluid liquid-crystalline *cis-trans* state which allows increased molecular motion and decreased molecular packing, resulting in a decreased membrane width. The *cis* double bonds of the unsaturated fatty acyl chains provide an angular conformation which disorders the bilayer lattice structure and decreases the hydrophobic interactions of the ester bond as well as the terminal methyl groups. The membrane becomes more disordered by the formation of a *trans-gauche* conformations in the fatty acyl chains which contain double bonds. The unsaturated fatty acyl chains possess more motional freedom than the straight stretched saturated fatty acyl chains.

5) The uptake and incorporation of unsaturated fatty acids (linoleic acid) and its acylation in the -position of glycerophospholipids (Hegner, 1980).

The special physicochemical properties of the membrane lipids play a significant role in modulating the function of membrane proteins such as:

1) membrane associated transport or cotransport systems. For example, carrier-mediated glucose transport or the cotransport of Na^+ and amino acids.

2) membrane-bound enzyme activity; for example, Na^+K^+ ATPase.

The positive and/or negative charges of the phospholipid polar head group interact with the surrounding proteins and may influence protein activity. The length of the hydrocarbon chains on the phospholipid groups also affect the activity of the proteins. The boundary lipid around membrane proteins may influence their function. The lipid in this microenvironment may have physical properties different from those of the bulk lipids (Figure I-2).

The presence of proteins in the membrane has been demonstrated not to affect the fluidity of the BBM at physiological temperature (Brasitus et al, 1979; Brasitus and Schachter, 1980; Mutsch et al, 1983).

The fluidity of brush border membranes has been investigated in rats (Brasitus et al, 1979, 1980, 1984; Schwarz et al, 1985; Brasitus & Schachter, 1980; Schachter & Shinitzky, 1977) and rabbits (Schwarz et al, 1984; Mutsch et al, 1983). Brush border membranes are less fluid than many other biological membranes, due to the unusually high ratio of cholesterol to phospholipid. The low fluidity of brush border membranes has been suggested to have functional importance:

1) Efficient enterocyte nutrient and electrolyte transport (Papahadjopoulos et al, 1973; Schachter and Shinitzky, 1977).
2) Optimal conformation of surface receptors and enzymes (Shinitzky and Inbar, 1976).

Passive and carrier-mediated transport processes depend on the nature of the BBM hydrocarbon chains, the charge of the phospholipid polar head groups, and the interaction beween phospholipid and cholesterol. Any changes in the BBM lipids may reflect an adaptive change in order to alter nutrient transport.

I-3. DEVELOPMENT OF THE SMALL INTESTINE[1]

Introduction

A brief review on the development of the small intestine has been included to offer an understanding of the anatomy of the intestine, the development of the intestine (especially the crypt/villus unit), as well as mechanisms and signals which regulate intestinal function, morphology and brush border membrane composition.

Anatomical Background

The small intestine is a muscular tube capable of digestion and absorption of dietary nutrients, and movement of this dietary residue to the large bowel. Anatomically, the intestine is divided into three segments: the duodenum, jejunum and ileum. Four layers make up the structure of the intestine:

1. serosal connective tissue
2. smooth muscle
3. lamina submucosa
4. mucosa

The fourth layer is the most important for the understanding of the digestive and absorptive function of the intestine. The mucosa consists of a layer of epithelial cells supported by the lamina propria which are seen as fingerlike projections called villi. The villus structure of the mucosa is illustrated in Figure I-3.

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1. A version of this chapter section has been published. Thomson, ABR, and Keelan, M. 1986. Canadian Journal of Physiology and Pharmacology 64:13-29.

The majority of the intestinal villus cells are enterocytes. Enterocytes are columnar in appearance and have a striated border which is thought to greatly improve the absorptive capacity of the intestine. This striated border is also known as the brush border membrane (BBM) or microvillus membrane (MVM), and lies in direct contact with the luminal contents. Other cells also populate the villus:

1. goblet cells: primary function is mucus secretion
2. endocrine cells: tuft or specialized M cells
3. lymphoid cells

The crypts of Lieberkuhn are located at the base of the villi, and are tubular in shape. Cells lining the crypts are more rounded in shape. Crypts provide the source of immature undifferentiated cells. These undifferentiated cells leave the crypt and develop into fully functional absorptive cells as they migrate up the villus. The migration takes approximately 2-3 days at which time the cells are sloughed from the villus tip into the lumen. The crypt constantly produces new cells in order to maintain villus structure and function. Another cell type is seen at the crypt base: Paneth cell.

The focus of these studies will be on the mature enterocyte. The apical membrane of the striated border is thicker (10.5 nm) than the unspecialized basolateral membrane (7.5 nm). The striated border consists of many microvilli approximately 0.1 μm wide and 1 μm long. One enterocyte has approximately 3700 microvilli, but this number varies depending on the species and type of calculation used, e.g. rat has 65 MV/μm^2; dog has 34 MV/μm^2 (Taylor and Anderson, 1972).

The effective absorptive surface per unit area is kept constant at 25 $\mu m^2/\mu m^2$ villus surface, therefore the microvilli increase surface area approximately 25 times. Cell maturity can be measured by function, enzyme activity, and microvillus ultrastructure. The microvilli of the villus tip cells are the longest (Brown, 1962; Ianan and Elian, 1976; Smith et al, 1983). Others suggest the midvillis is best for digestion, and absorption as judged by morphology (Merrill, 1967; Brunser, 1976; Phillips, 1979).

There is a "fuzzy" apical cell coat called the glycocalyx surrounding the microvillus tips made up of glycoprotein. The role of the glycocalyx has been suggested as a site of surface enzyme localization, and possibly regulates access to the membrane surface. The MVM is the site of many digestive enzymes which act at the cell surface; e.g. sucrase. The microvillus core contains actin-like bundles (Mooseker and Tilney, 1975) with myosin at the terminal web at the base of the microvilli. This core is thought to have a contractile capacity, possibly to help speed the diffusion of nutrients.

A normal mucosal villus morphology depends on the maintenance of a balance between cell renewal and cell loss. Villus morphology is very sensitive to changes in cell dynamics.

Pre-natal Development: Preparation for Suckling

As the discipline of developmental biology has advanced, the physiological and biochemical aspects of gastrointestinal ontogeny have overshadowed the morphological aspects. Morphogenesis and cytodifferentiation are normally congruent and may be controlled by the intracellular environment, the position of the cell relative to surrounding cells, or by molecules at the cell surface. At about the 14th day of embryonic life in the human, the intestinal tract begins to develop from the premature entoderm and the surrounding splanchnic mesoderm. A tube-like structure is formed, and by day 18 the primitive gut is lined with nondifferentiated cuboidal cells which proliferate, obliterate the lumen, and then the intestine is recanalized. In the human, specialized junctional complexes and secondary lumina develop during transition from stratified epithelia at 8-9 weeks to simple columnar epithelia at 9-10 weeks (Trier and Moxey, 1979). Well-defined crypts and villi are present by the end of the 12th week of gestation. Villi form by the seventh week and the crypts of Lieberkuhn form by week 12. During this period, active transport of glucose and amino acids occurs, and by week 20 the jejunal transport exceeds that observed in the ileum. Certain dipeptidases and disaccharidases appear. The intestinal changes in length and weight are coordinated with an increase in many striking biochemical parameters. The growth of the intestine is similar to that of the whole organism, and follows a sigmoid pattern which reaches a plateau when maturity is attained. In early gestation the small bowel of the human fetus develops from a short hollow tube lined with squamous epithelium, to an intestine which is elongated approximately 1000-fold and a mucosa composed of well defined villi and crypts. Ultrastructural studies show that the villus epithelial cells have well-defined microvilli and glycocalyx and the terminal web is apparent by the second trimester of pregnancy (Varkonyi et al, 1974). In rodents, the small intestine matures structurally and functionally during the last few days of gestation at which time enzyme activities continuously increase along the entire intestinal mucosa (Hohn et al, 1977; Calvert et al, 1979). By the seventh month of intrauterine life, the human fetus has the biochemical and physiologic capacities for limited digestion and absorption (Henning and Guerin, 1981; Sunshine et al, 1971).

The effect of intrauterine malnutrition on gastrointestinal development in humans is unknown. In rats intrauterine growth retardation leads to a decrease in both intestinal and pancreatic weight, due to a reduction in the number of cells (Lebenthal et al, 1981a,b), as well as to a reduction in brush border membrane (BBM) enzyme markers, and a decrease in villus height, crypt depth and epithelial cell migration. When the pregnant dam is fed a low-protein diet during gestation, there is a reduction of pancreatic acinar tissue and of exocrine enzymes (Klotz et al, 1972) in the offspring. When rat pups are malnourished during the suckling period, pancreatic enzyme and BBM activities fall (Patlak et al, 1981). The intestinal uptake of glucose and glycine may be increased in these malnourished animals (Patlak et al, 1981). Studies performed in severely malnourished children have also demonstrated reduction in the secretory capacity of the pancreas (Barbezat and Hansen 1968), blunting of the villi and elongation of the crypts, and lactose and sucrose intolerance (Patlak et al, 1981).

Thus, in the human, anatomic differentiation of the fetal gut resembles that of the newborn infant by 20 weeks' gestation, but functional development is limited prior to 26 weeks. This development may be influenced by the nutritional state of the fetus: intrauterine or post-partum malnourishment alters the normal functional and morphological maturation of the intestinal tract.

Post-Partum Development: Preparation for Weaning

Morphological Development of Crypt/Villus Unit

In the rat, the length of the gut achieves 90% of its adult value by day 40, whereas body weight increases in a linear fashion (Buts and

DeMeyer, 1981). In each segment of the small bowel, the increase in the weight of the mucosa and of the total small bowel was greatest between suckling and weanling/growing rats. For each segment of intestine, differences in DNA were also maximal between suckling and weaned rats (Buts and DeMeyer, 1981). Between 20-40 days of age the body weight of the animals continued to rise, but the ratio of the small intestine to body weight falls (Calvert et al, 1981).

The basic functional unit of the intestine is the crypt/villus unit. The cells proliferate in the crypt. The entire proliferative cycle of an intestinal crypt cell is about 1 day, and the life span of a villus cell is about 2 days. Mitosis lasts 1 hour and DNA synthesis lasts 12 hours. In addition there is a 2-hour pause between the cessation of DNA synthesis and the beginning of mitosis (G_2), and another pause of about 9 hours between mitosis and the onset of DNA synthesis (G_1). Usually each villus is supplied with cells from about three crypts. The exact number depends on the species. At the termination of proliferation in the upper third of the crypt, differentiation is initiated. The cells migrate out of the crypt and onto the villus, and in humans these cells are finally extruded from the tip of the villus 48 to 72 hours later. Differentiation is distinguished by morphologic and chemical changes. As a result of this process, the epithelial cells of the villus are endowed with the specific functions required for digestion and absorption. The life cycle of the cell terminates when the cell is sloughed from the tip of the villus. Up to 14 days in the post-natal period in the rat, enzyme activities are similar to those observed in the last few days of the prenatal period. By the postnatal day 15 to 24, a period of adaptation begins in which the mucosa undergoes a transition into the stage of adult maturity both enzymatically and morphologically. Chemodifferentiation is complete by day 24. The results of these studies by Hohn and coworkers (1977) suggest that chemodifferentiation is synchronized with morphological changes in the intestinal epithelia. Although the crypt/villus unit begins to develop in utero, only in the post-natal period do villi increase in number and height (Murphy and Daniels, 1979). The number of villi increases to the age of 3 months while the number of crypts increase to the age of 8 months (Kapadia and Baker, 1976). In addition, the mean length of the villus base increases up to the fifth month, at which time the villi have changed from a cylindrical shape to a ridge shape.

The crypt cells secrete large quantities of fluid but are not developed for digestion and absorption. The enzymes involved in these processes increase markedly as cells migrate up the length of the villus (Fortin-Magana et al, 1970). With maturation of the enterocyte, the mitochondria become more numerous and elongated, and the microvilli increase in length (Pearse & Riecken, 1967).

There is only limited data on the rate of cellular turnover which occurs with early development In fetal rat intestine, in contrast to that of neonates and adults, proliferation occurs along the entire length of both crypts and villi (Hernell et al, 1981). In adults, enterocyte microvilli mature as the cells produced in the crypts migrate up the intestinal villi. In contrast, in the fetus, maturation of the microvilli is not necessarily associated with cell migration along the villi (Hernell et al, 1981). During the early formation of the villus, all the epithelial cells are capable of dividing (Deren, 1968). The number of villi in the rat does not change with age, but the height of the villi may fall (James et al, 1982). At weaning the microvilli become longer and thinner, maintaining the same volume but larger surface area (Dunn, 1967). In the rat, cell migration in the suckling period is slower than at maturity, although this is not true for pig or guinea pig (Herbst and Koldovsky, 1972; Koldovsky et al, 1966). The mitotic index of rat crypt cells, the labeling index following the injection of tritiated thymidine, and the depths of the intestinal crypts increase during the weaning period (Herbst and Koldovsky, 1972). This four- to five-fold increase in jejunal and ileal turnover which occurs at weaning, and the enhanced cell proliferation, lead to mucosal hyperplasia. In man, the rate of mitosis, cell turnover, and migration have been studied only in a single anencephalic newborn child (Herbst and Koldovsky, 1972). The rate of cell migration from crypt to villus was less than that found in mature human intestine (Lipkin et al, 1963).

Enzymatic and Transport Development of Crypt/Villus Unit

The rat small intestine undergoes an array of morphological and biochemical changes during the third postnatal week (Henning et al, 1979; Henning and Guerin, 1981; Moog 1979, 1981). Neonatal rats have no detectable sucrase, low maltase, and high levels of lactase and acid B-galactosidase activities in their small intestine. PVP uptake, reflecting pinocytosis, parallels the fall in lactase (Henning and Guerin, 1981). The activities of sucrase and maltase increase abruptly about the 15th day of age. These two enzymes continue to rise during the third and fourth week, with a concomitant decrease in lactase. The third week of life in rats corresponds to the weaning time. This is marked by a shift from a fat-rich diet supplied by the mother, to a carbohydrate-rich diet supplied by laboratory chow. The carbohydrate is also changed from an almost exclusively lactose-containing milk, to a predominant glucose polymer- and sucrose-containing chow. Thus, a temporal relationship exists between dietary changes and the increase in brush border membrane activity of sucrase and maltase, with a corresponding decrease in lactase activity.

The brush border exists in a steady-state, governed by the rates of synthesis and degradation of labile proteins (Jarvis et al, 1977). Many changes occur at the time of weaning when the intestine takes on many of the properties of the adult intestine. These changes in enzymatic and transport function can be categorized into two broad groups (Henning et al, 1979): activities that are high at birth and then decline, e.g. lactase and glucose and amino acid absorption (Shepherd et al, 1980), and activities that are absent or low at birth and appear or increase at weaning, e.g. sucrase, maltase, isomaltase, alkaline phosphatase, Na^+K^+ATPase, and Na^+ transport (Shepherd et al, 1980). This grouping of changes may be further subdivided, depending upon the subsequent developments which occur with aging.

As the epithelial cell population expands during the growth of the organism, the performance of various functions will also change. For example, with an increase in the surface area of the microvilli, some functions may increase due to the expansion of the surface area. Expressing enzyme activities or transport results on the basis of the whole animal does not provide an estimate of the change in the functional capacity per unit of intestinal tissue. Expressing results on the basis of intestinal weight may be also inappropriate since the wall doubles in thickness at the time of weaning. In addition, the proportion of the intestinal wall comprised of mucosa also changes. Perhaps the best manner of expressing the results would be on the basis of function per unit number of cells. However, it is often the relative and the qualitative rather than the quantitative aspects which direct attention - the decline in lactase but increase in sucrase in the postpartum period, for example.

Proximal-Distal Gradients

There are regional differences in enzymatic activities along the brush border of the small intestine during postnatal development and in the adult (Antonowicz and Lebenthal, 1977; Ecknauer and Raffler, 1978; Menard and Malo, 1978). In the mouse, the establishment of these regional differences in brush border enzymatic activities occurs during fetal development and before lactation (Calvert et al, 1981). From day 16 to 19 of gestation of the mouse, the height of the duodenal microvilli increases on the absorptive cells located near the tip of the villi, and the number of microvilli per unit area also rises, increasing the microvillus surface area by a factor of approximately five-fold. Lactase, glucoamylase and alkaline phosphatase appear between days 16 and 18 of gestation. Thereafter all three enzyme activities increase rapidly, and a decreasing gradient of activity is established from the proximal to the distal segment of the small intestine. In the rabbit, the histological maturation of the intestine occurs 20 days after birth, and at all stages morphogenesis follows a cranio-caudal gradient developmental pattern (Toofanian and Targowski, 1982). Proximal-distal morphological gradients are established within the first month after birth in the rat. Villus height and crypt depth are greater in the proximal than the distal intestine. As a result, the villus mucosal surface area is greater proximally than distally (Kapadia & Baker, 1976).

The ileum of the newborn rabbit is similar to that of the adult in its ability to actively absorb Na^+, Cl^- and alanine, but differs from the ileum of the adult by having a greater passive permeability to ions and to amino acids (Cooke and Dawson, 1978). In the early postpartum period, the disaccharidase specific activities in the ileum remain constant whereas those in the proximal intestine continue to increase (Antonowicz and Lebenthal, 1977).

The jejunoileal gradient of villus size may be programmed *in utero*. Fetal intestine exhibits autonomous differentiation of structure and function when removed from its normal location late in gestation: fetal intestine planted into a synegeneic adult host grows, develops functions, and develops topographic specializations of the implanted segment (Kendall et al, 1977, 1979). Although food intake increases during lactation, this is unlikely to be the only controlling factor. However, the level of enzyme activity in the jejunum may be influenced by bacterial contamination as well as by bile and pancreatic secretions (Alpers and Tedesco, 1975). Thus it is likely that regional differences are partially under genetic control, and may be modulated further by environmental factors.

Regulation of Adaptive Changes in the Young: Mechanism and Signals

There is a degree of coordination between the maturation of digestion, absorption and metabolism in both laboratory animals (Henning et al, 1975a, 1979; Koldovsky, 1979; Mills and Davies, 1979; Moog, 1979) and in humans (Grand et al 1976, 1979). The fundamental question is as yet unanswered: What controls this adaptive process? The search for signals which might trigger the intestinal developmental changes has centered on factors which themselves are known to change at or just preceding the intestinal developmental changes. Let us consider the possible signals.

a. Genetic Endowment

The genetic endowment is contained in the base sequence of the DNA molecules and controls the differentiation of specific tissues and cells. This differentiation proceeds independently of exogenous factors. For example, segments of small intestine from fetal rats develop normally after implantation in the kidney, in the subcutaneous space or when cultured *in utero* (DeRitis et al, 1975; Kendall et al, 1979; Leapman et al, 1974). The changes in mucosal disaccharidases parallel those found with *in situ* development (Kendall et al, 1979). This genetic endowment may vary by way of mutation or as the result of evolutional selection. There are a number of examples. First, in congenital sucrase-isomaltase deficiency, the enzyme complex in the brush border of mature epithelial cells of the small intestine is greatly reduced or absent (Gray et al, 1976). An abnormal protein is found in the cytoplasm of these enterocytes, which may represent a modified enzyme complex in the brush border membrane (Schmitz et al, 1980). Secondly, glucose-galactose malabsorption arises from the genetically-determined lack of the hexose carrier (Eggremont and Loeb, 1966; Meeuwisse and Dahlqvist, 1966).

b. Environmental Influences

i. Dietary Constituents

Dietary constituents are important in modifying the developmental pattern of intestinal and pancreatic enzymes (Figure 3). At the time of weaning, the dietary content of fat and lactose falls, and the dietary content of sucrose and starch rises. Thus, weaning is associated with a change from the relatively high-fat low-carbohydrate diet of milk, to the relatively low-fat high-carbohydrate diet of solid foods. The effect of different types of milk on intestinal transport development has not been established in animals or in man. Studies in adult animals have shown that some specific digestive functions of the small intestine can be affected by the type of food ingested. For example, maltase and sucrase but not lactase increase within 5 days when man or rats are fed a diet high in either maltose or sucrose (Lebenthal et al, 1972). Lactase does not appear to be an adaptive enzyme in man (Knudsen et al, 1968), although feeding lactose increases brush border membrane lactase in rats (Lebenthal et al, 1973). When suckling rats are fed a diet containing cow's milk protein to which has been added lactalbumin and corn oil to approximate the protein and fat concentration of rat milk, the activities of jejunal sucrase and maltase, the relative weight of the intestine, the mitotic index of crypt cells, and the rates of cellular proliferation and migration are all altered to approach that of the adult animal (Herbst and Koldovsky, 1972). Furthermore, the feeding of a high-carbohydrate diet to adult rabbits increases their jejunal uptake of glucose (Thomson and Rajotte, 1983c).

Are the changes in intestinal sucrase and lactase due to changes in the cell cycle, with a new population of mature cells emerging from the crypts of Lieberkuhn? The digestive enzymes are on the outer surface of the brush border membrane (Hayashi and Kawasaki, 1982) and the process of packaging and export may be sites of molecular control mechanisms (Cezard et al, 1979). It is presumed that interaction between the epithelium and mesenchyme is necessary for development and differentition of the small intestine. Thus, changes in cell proliferation, differentiation of individual cell functions, or the rate of cell loss from the villi may alter the function of the crypt-villus unit. It is also possible however that the diet-related changes in enzymes may be due to altered activities within the differentiated epithelial cells already on the villi (Hayashi and Kawasaki, 1982; Henning et al, 1975b).

Finally, although the developmental pattern of lactase, glycoamylase and alkaline phosphatase have many similarities, the activity of one enzyme such as lactase may increase much faster than the other two at a given point in time (Calvert et al, 1981). This suggests that some factors may modulate selectively the developmental pattern of each enzyme, and that the changes are not due just to morphological growth.

Feeding premature infants a high-fat diet has no effect on pancreatic enzymes (Zoppi et al, 1972), but feeding a high-protein diet results in higher trypsin and lipase levels in the pancreatic secretions. Feeding a high-starch diet results in the appearance of low levels of amylase activity. As compared with premature infants fed milk-based formula, those infants fed a soy-based feed had a greater pancreatic secretion of trypsin and lipase (Lebenthal et al, 1981a).

Oral intake and body weight, rather than just chronologic age, may act as the physiologic trigger for postnatal maturation of the small intestine. Increased oral nutrient intake and accelerated weight gain leads to precocious maturation of the small intestinal epithelium (Gall and Chung, 1982). In animals with accelerated weight gain, there were lower unidirectional Na^+ fluxes, lower glucose-stimulated Na^+ absorption, lower fluxes of glucose, reduced lactase and thymidine kinase activities, and higher sucrase activity in the brush border membrane. This transport and enzyme profile in the jejunum from the heavier accelerated-growth suckling rabbits is characteristic of the intestinal epithelium from more mature animals.

When the diet of the lactating rat is sufficiently restricted that the suckling animals become malnourished (Hamilton et al, 1983), normal mucosal enzyme developmental patterns are delayed. The incorporation of ^3H-thymidine into the crypt cells was less, suggesting a lower rate of proliferation. These abnormalities were quickly reversed by refeeding. Many of these findings in the malnourished suckling rat were similar to those previously reported in more mature animals after severe protein-calorie malnutrition (Viteri and Schneider, 1974).

It is possible that dietary fat will influence the brush border membrane lipid composition and will thereby alter enterocyte enzyme activity and transport function. Indeed, active and passive transport is altered with aging, diabetes, species, and with dietary manipulation (Thomson, 1984d), and each of these experimental maneuvers is associated with changes in membrane enzymes and phospholipids (Keelan et al, 1985; Brasitus et al, 1984). Differences in membrane lipid content or distribution are associated with altered enzyme activities and membrane physical properties, as defined by the temperature of the lipid phase transition, as well as by differences in the Arrhenius activation energy of enzymes (Sanderman, 1978). For example, insulin receptor sensitivity (Ginsberg et al, 1982) is influenced by the fatty acid, phospholipid and cholesterol content of the membrane under consideration. Using a bacterial system, Read and McElhaney (1975) showed that the rate of glucose uptake was influenced by the fluidity of the membrane lipids, which in turn was influenced by the lipid content of the membrane. These changes in membrane function as a result of alterations in the diet occur relatively rapidly (Thomson and Rajotte, 1983c).

The intestinal brush border membrane exhibits a lipid phase transition (Brasitus et al, 1980; Brasitus and Schachter, 1980). Membrane fluidity will be influenced by cholesterol, by the type and amount of phospholipids, the number of unsaturated double bonds within the phospholipid acyl chains, and by the presence of other amphipathic substances such as proteins. The membrane proteins are themselves of two major types: extrinsic, such as sucrase, lactase, and maltase; and intrinsic, such as glucose transport, and calcium- or magnesium-ATPase. The functions of these intrinsic proteins showed a discontinuation of the Arrhenius plot (Brasitus et al, 1980), suggesting modulation by the fluid state of the lipid. Furthermore, changes in the lipid content of the membrane are also associated with changes in the brush border membrane activity of sucrase, alkaline phosphatase, and glucose uptake (Keelan et al, 1985). Thus, it is reasonable to speculate that some of the age-related changes in intestinal function are related to alterations in the brush border membrane lipid content.

In adults, changes in the type or the amount of fiber in the diet may influence the intestinal absorption of nutrients (Schwartz and Levine, 1980; Freeman, 1984), and alter intestinal structure (Morin et al, 1980; Sircar et al, 1983). The effect of the introduction of fiber into the diet on the development of the intestinal tract has not been reported.

The topic of the development of the infant gut flora has been reviewed (Borriello and Stephens, 1984). Much of the recent work has centred on investigations into the relationship between the type of infant feed, such as breast milk or formula preparations, and the ecology of the intestine (Hewitt and Rigby, 1976; Willis et al, 1973). At birth the gastrointestinal tract is sterile, but colonization with aerobes and anaerobes begins shortly after birth (Borriello, 1981). Breast-fed infants have fewer putrefactive organisms, few E-coli but more counts of lactobacilli than do infants fed by bottle. These differences tend to persist until weaning when the fecal flora becomes more complex and similar in the two groups of infants, and assumes the characteristics of the adult flora by four years of age.

The activities of alkaline phosphatase, maltase and lactase of the duodenum were higher in germ-free than in conventional rats, and were decreased with aging in both types of animals (Yazawa et al, 1981). Serum cholesterol at the age of 8 and 40 weeks and serum triglyceride at the age of 40 weeks were higher in germ-free than in conventional rats (Kawai et al, 1981). Intestinal microflora tended to depress the age-related increase of serum triglyceride and lipase activity of the pancreas and the duodenal and jejunal contents. This suggests that there are influences of indigenous intestinal microflora and of aging on the activities of various enzymes in the gastrointestinal tract.

The possibility of a dietary influence on intestinal function is further strengthened by the observations that sucrase activity rises precociously with the early feeding of sucrose (Raul et al, 1978). In addition, prolonged nursing (i.e. non-weaning) leads to a slower decline in lactase activity (Lebenthal et al, 1973). In contrast, prolonged nursing does not influence maltase or sucrase activities (Henning, 1982; Lebenthal et al, 1973), although the total activity of sucrase may be reduced, with no effect on the time of onset on this reduction. When rat pups are weaned early on day 17, there is an immediate increase in intestinal length, decrease in lactase activity, and a precocious increase in sucrase and maltase (Lebenthal and Lee, 1983). In contrast, when pups are nursed up to 25 days, they showed no significant delay in the increase in sucrase and maltase, and no change in the persistently higher level of lactase. At 19 days of age, early weaned pups had serum levels of corticosteroids about three times that of control or prolonged nursed pups. These results are in agreement with experiments using intestinal explants (Kendall et al, 1977,1979) or using in vitro cultures (DeRitis et al, 1975). There is further evidence that these dietary alternatives are not, by themselves, totally responsible for the functional changes which occur at weaning: 1) premature weaning does not alter the rate of decline of pinocytotoic absorption of protein (Halliday 1957); 2) oral administration of sucrose to suckling rats has no effect on the developmental pattern for sucrase (Rubino et al, 1964); and 3) prolonged suckling has no effect on variable aspects of function, and the prevention of weaning has little effect on total enzyme activity, as is the case for maltase (Lebenthal et al, 1973) and alkaline phosphatase (Lichtenberger and Johnson, 1974). Prolonged suckling may be associated with a slight delay in the onset of maturation of gastrin receptors (Peitsch et al, 1981; Takeuchi et al, 1981), of lactase (Lebenthal et al, 1973; Lichtenberger and Johnson, 1974), and of pancreatic enzymes (Dumont et al, 1978), or may be associated with a reduced activity of sucrase (Henning, 1980), but has no effect on the time of its onset.

However, some influence of intraluminal factors certainly exists: Tsuboi et al (1981) performed surgical bypass of ileal segments of suckling rats and followed subsequent intestinal development. The bypassed segment, although achieving normal maturational patterns of active sucrase appearance and maltase accumulation, continued to maintain coincident immature patterns of high lactase activity and low cell turnover times. The intestine in continuity showed precocious appearance of active sucrase and a decline in lactase activity and enterocyte life-span. Thus it would appear that the dietary change of weaning may modulate the developmental process, but although dietary factors may be important, alone they do not provide the trigger for the ontogenic changes. It is also possible that systemic release of gastrointestinal hormones or growth itself may lead to accelerated maturation of the central nervous system, which then stimulates the hypothalamus-pituitary-adrenal axis. Many of the biologically active peptides may have a dual role as gut hormones and neuro-regulatory agents (Dockray, 1979). Thus dietary changes appear to have some influence on the time of onset or extent of developmental change of the intestine.

ii. Hormones

Oral nutrients may act directly on the intestinal tract, or may act indirectly through an effect of hormones in response to food ingestion (Figure 3). The timing of these changes in intestinal development is associated with the process of weaning, but it is controversial whether the transition to solid food plays a causative role (Henning and Guerin, 1981). On the other hand there is evidence which shows that intestinal maturation is hormonally mediated. The principal candidates for this hormone signal are cortisone and thyroxine (Koldovsky, 1979; Moog, 1979). The administration of gluccorticosteroids or thyroxine to suckling rodents before a critical stage of maturation causes elongation of the microvilli, and an increase in some of the brush border membrane enzymes (Lebenthal et al, 1972; Lebenthal, 1977). Adrenalectomy or thyroidectomy abolishes the normal pattern of postnatal development. Rat milk contains a variety of hormones and growth factors (Koldovsky, 1980), including thyroxine, TSH and ACTH (Koldovsky et al, 1980; Vigouroux et al, 1980). By suckling rat pups with dams which had been lactating longer than controls, Henning (1982) showed that sucrase (an enzyme which is undetectable until postnatal day 17 and then rapidly rises to adult levels of activity) was higher in the pups cross-fostered to the longer-lactating dams. Analysis of stomach contents showed that these pups had almost completely weaned by day 21. Thus neither the appearance of jejunal sucrase nor the onset of weaning was cued by the appearance of a factor in the milk, but the lactational stage of the dam did affect the rate of completion of weaning and the terminal portion of the sucrase rise.

iii. Glucocorticoids

There have been many studies of the role of glucocorticoids in postnatal development (Ballard, 1979). Several functions have been shown to mature precociously after administration of glucocorticoids or ACTH, or for development to be delayed or arrested by adrenalectomy (Table I-1). For example, the activities of duodenal phosphatase (Moog, 1962), jejunal sucrase (Doell et al, 1965), and maltase, nonspecific esterases, and pancreatic and intestinal lipase are all precociously induced in suckling animals following the injection of glucocorticosteroids, although not all workers have been able to confirm an effect of corticosteroids on promoting the normal maturation of jejunal lactase and alkaline phosphatase (Kedinger et al, 1980; Simon et al, 1982).

Steroids also precociously induce the ability of the intestine of the suckling animal to transport glucose against a concentration gradient, to decrease the ability of the columnar epithelial cells of the jejunum and ileum to absorb protein intact (Clark, 1959), and to increase the rate of cellular proliferation and migration of the intestine of the suckling but not of the adult rat (Herbst and Sunshine, 1969). Glucocorticoids have been shown to accelerate the development of various intestinal mucosal enzymes (Henning, 1978; Moog et al, 1973), and ileal vitamin B_{12} receptors (Gallagher and Foley, 1972). The concentration of intestinal glucocorticoid receptors in the rat peaks at the time of weaning (Henning et al, 1975a). Glucocorticoids also stimulate digestive and absorptive functions in the adult rat. While the adrenal corticosteroids are potent determinants of the rate of developmental changes of intestinal hydrolases, these hormones are probably not necessary for these enzymes to eventually reach adult activities (Martin & Henning, 1984).

Why do these glucocorticoid-mediated changes in the rodent occur specifically in the third postnatal week? Both total and free corticosterones exhibit a dramatic surge in plasma concentration (Henning, 1978) beginning on approximately day 14, just before the postnatal intestinal developmental changes occur. What is the mechanism by which glucocorticoids cause intestinal maturation? The two theories suggest the synthesis of new protein, or the reorganization of existing enzymes (Moog, 1971). Thus, the temporal sequence of an increase in serum corticosterone before that of sucrase in the brush border membrane suggests a physiological role for the hormone in developmental maturation.

The developmental pattern of the human infant differs from that seen in animals. The activity of all disaccharidase enzymes is high at birth and declines over the first few months of life, while alkaline phosphatase activity which is low at birth gradually increases (Dahlqvist and Lindberg, 1966). No information is available on the hormonal control of human intestinal development in early life. However, corticoids may play some role in human intestinal maturation, as suggested from the finding that infants of mothers who had received adrenocorticoids during the final stages of pregnancy were found to have higher bile salt pool sizes, synthesis rates, and intraduodenal conentrations of bile acids when compared to premature infants born of untreated mothers (Watkins, 1981).

iv. Thyroid Hormones

Both total and free thyroxine have received attention as the possible trigger for developmental changes in infant rats, because the circulating concentration of thyroxine as well as the circulating level of corticosterone rise during the second postnatal week (Dussault and Labrie, 1975; Kumegawa et al, 1980). Hypothyroidism prevents or delays intestinal maturation, and this effect is reversed by giving physiological amounts of T_3 or T_4. Giving thyroid hormones during the first or second postnatal week may result in precocious development of the intestine. The thyroid hormones may exert their effect by causing an increase of plasma cortisone (Malinowska et al, 1974), or by changing feeding patterns (Wakelam et al, 1979). The possible roles of thyroxine are summarized in Table I-2: T_4 may play a permissive role for the increase in sucrase (Martin & Henning 1982) and pancreatic amylase (Kumegawa et al, 1980), or a primary role for the decrease in lactase (Yeh and Moog, 1974), or the increase in alkaline phosphatase, parotid amylase, hepatic glucokinase, and corticosteroid binding globulin (Yeh and Moog, 1978), and a shared role for lysosomal hydrolases (Jumawan and Koldovsky, 1978; Koldovsky et al, 1974) and for pinocytosis (Moog and Yeh, 1979). On the other hand, corticosteroids may play a permissive role for the increase in parotid amylase, and a primary role for the increase in intestinal sucrase, intestinal sodium transport, pancreatic amylase, and bile salt metabolism (Guiraldes and Hamilton, 1981; Guiraldes et al, 1981; Henning, 1981). Thus, thyroid and adrenal function are important in the normal pattern of postnatal development (Henning, 1981; Watkins, 1981), with the postnatal increase in serum T_4 concentrations likely providing the cue for the developmental rise of both corticosteroid-binding globulin and corticosterone in the rat (D'Agostino et al, 1982).

v. Other Factors

Epidermal growth factor given to pregnant mice increased the brush border membrane alkaline phosphatase and trehalase activities in the 18 day gestation offspring (Calvert et al, 1982). The administration of insulin was able to provoke the premature appearance of intestinal sucrase activity in the suckling mouse (Menard and Malo, 1979). This effect is rapid and suggests that cells already on the villus may be responsive to insulin. Another possible neural control mechanism that remains to be adequately explored is the sympathetic nervous system which appears to influence the circadian rhythm of crypt cell division and migration (Herbst and Sunshine, 1969; Klein, 1981). Thus it is possible that insulin, epidermal growth factor, glucocorticosteroids, thyroxine, as well as other as yet unidentified hormones and gastrointestinal peptides may play a role in the intestinal developmental process. Finally, changes in the activity of ornithine decarboxylase activity have been described with aging (Ball and Balis, 1976), and the importance of polyamines in the development of the intestine needs to be established.

Summary

Different patterns of development continue after birth, and a number of general trends are apparent: 1) activities that are high at birth, and then continue to decline, e.g. the intestinal uptake of fatty acids and cholesterol; 2) activities that are high at birth, decline, and then remain unchanged, e.g. lactase; 3) activities that are high at birth, decline, and then increase with aging, e.g. the transport of glucose and amino acids; 4) activities that are low at birth, then continue to increase with aging, e.g. intestinal unstirred layer resistance; 5) activities that are low at birth, then increase, e.g. sucrase, maltase, isomaltase, alkaline phosphatase, Na^+K^+ATPase and Na transport; and 6) activities that are low at birth, increase, and then decrease again, e.g. mucosal surface area. Whereas the transport of glucose and amino acids, and the intestinal mucosal surface area "regress" to weanling levels, the activity of lactase falls and sucrase rises, but then remain stable after some arbitrary point. Finally, some processes progressively change with time, e.g. the intestinal uptake of fatty acids, cholesterol, and fatty alcohols. It is unknown whether these latter processes continue to change with age, or whether they eventually reach a plateau. What then are the factors responsible for the "breaking" of the changes, leading to the leveling of activity? What are the factors responsible for the reversal of a falling or an increasing trend? Are the signals different for the three trends: continuation, plateau, and reversal? The remarkable degree of coordination between the development of various aspects of gastrointestinal function suggests that the processes may be signalled by a small number of factors, such as weaning, nutrient intake, growth, and hormones. However, these three distinct patterns - continuation, plateau and reversal - strongly suggest that several inter-related signals are at work during the aging process. Furthermore, each of these processes such as transport, enzyme activity and morphology may be altered in health and in disease, but the direction of changes is not necessarily congruous.

I.4 AIMS AND SCOPE OF RESEARCH

The aims of this research proposal are to:

1) test the hypothesis that intestinal adaptation occurs structurally and biochemically via changes in villus morphology, and brush border membrane (BBM) enzyme and lipid composition.
2) determine whether a relationship exists among intestinal transport function, villus morphology and BBM composition (Figure I-4).

The intestinal BBM enzyme and lipid composition, as well as villus morphology will be defined in each of the following animal models:

1) aging
2) diabetes mellitus and fasting
3) chronic ethanol consumption and food restriction
4) external abdominal irradiation

These models have been chosen for study since the functional adaptation of the intestine has been previously reported.

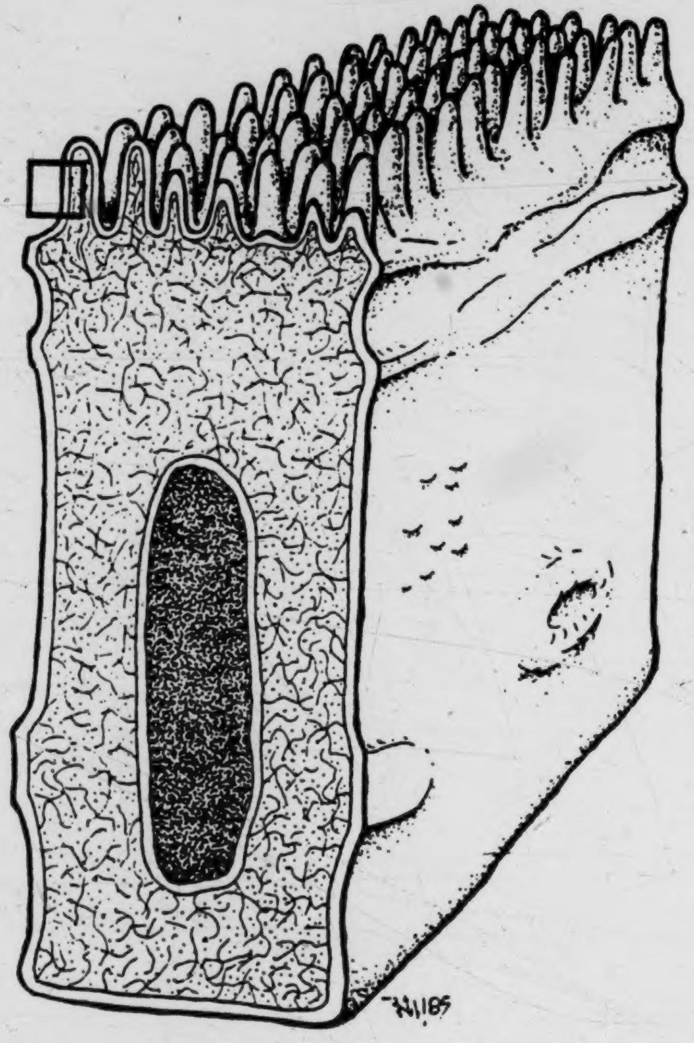

Figure I-1. Enterocyte illustrating the brush border membrane.

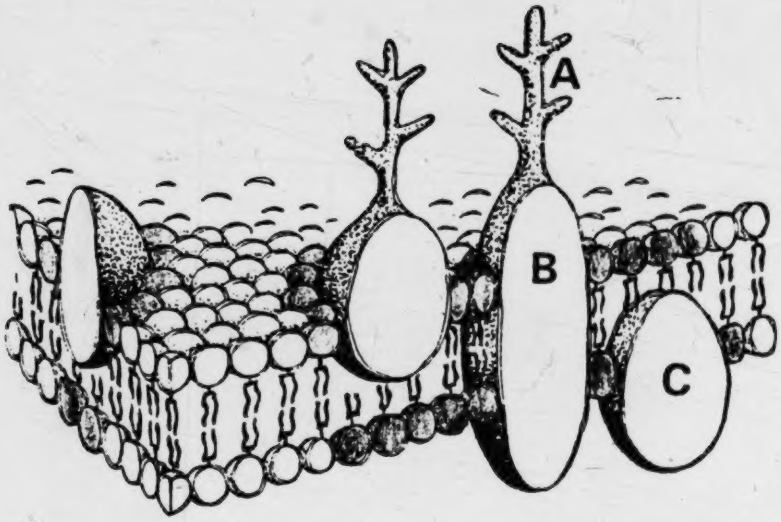

Figure I-2. Model of membrane lipid bilayer. Intrinsic (B) and extrinsic (C) proteins are embedded in the lipid bilayer. A carbohydrate moiety (A) may be bound to the membrane proteins. The shaded area illustrates the boundary lipid around the membrane proteins.

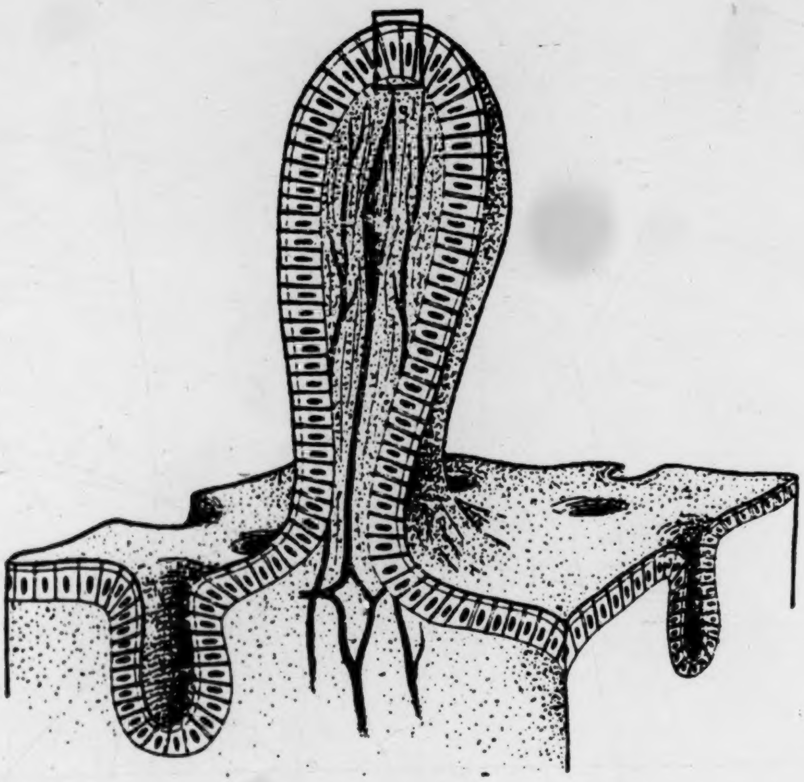

Figure I-3. Villus structure. The box illustrates the enterocyte.

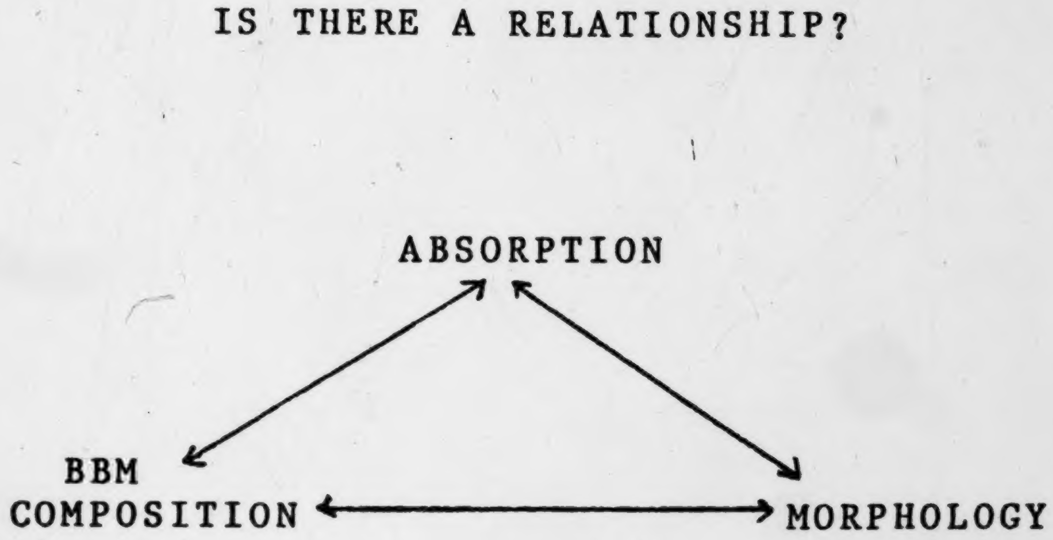

Figure I-4. Is there a relationship among intestinal transport function, villus morphology and BBM composition?

Table I-1. CHANGES IN POSTNATAL SMALL INTESTINAL DEVELOPMENTAL FUNCTION AS INFLUENCED BY GLUCOCORTICOIDS

Enzymes	lipase (Koldovsky et al, 1966)
	duodenal alkaline phosphatase (Moog, 1962)
	jejunal sucrase (Doell et al, 1965)
	maltase (Galand and Forstner, 1974)
	lactase (Raul et al, 1978)
Transport	jejunal glucose transport (Koldovsky et al, 1966)
	ileal pinocytosis (Daniels et al, 1973a)
	ileal B_{12} receptors (Gallagher and Foley, 1972)
	ileal bile salt transport (Little and Lester, 1980)
	"closure"; decreased absorption of immunoglobulins and other macromolecules (Clark, 1959)
	cellular proliferation and migration (Herbst, 1969)

Table I-2 IMPORTANCE OF THYROXINE AND CORTICOSTEROIDS IN INTESTINAL DEVELOPMENT

Thyroxine	Primary	Decrease	lactase
		Increase	alkaline phosphatase, parotid amylase, hepatic glucokinase, corticosteroid-binding globulin
	Permissive	Increase	intestinal sucrase and pancreatic amylase
Corticosteroids			
	Primary	Increase	intestinal sucrase, intestinal sodium transport, pancreatic amylase, bile salt metabolism
	Permissive	Increase	parotid amylase

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CHAPTER II.

EFFECT OF AGING ON INTESTINAL MORPHOLOGY, MARKER ENZYMES AND LIPID CONTENT OF BRUSH BORDER MEMBRANES FROM RABBIT JEJUNUM AND ILEUM[1]

INTRODUCTION

Aging is associated with changes in the intestinal absorption of hexoses, fatty acids, fatty alcohols, cholesterol, and vitamin D_3 (Bowman and Rosenberg, 1983; Hollander and Morgan, 1979a,b; Holt and Dominguez, 1981; Jakab and Penzes, 1981; Klimas, 1968; Pelz et al, 1968; Thomson, 1979, 1980a,b, 1981). Aging is also associated with a decreased incremental change in free energy of transfer, associated with the intestinal uptake of medium chain-length fatty acids, and a decreased rate of uptake of cholesterol in the rabbit (Thomson 1981) but increased uptake of cholesterol in the rat (Hollander and Morgan, 1979b). Membrane function may be influenced by membrane composition and fluidity (Cooper et al, 1975; Cooper, 1977; Cooper and Shattil, 1981; Grunge and Deuticke, 1974; Kroes and Ostwald, 1971; Read and McElhaney, 1976; Shattil and Cooper, 1976; Van Deenen et al, 1972) but there is a limited number of studies of fluidity (Brasitus et al, 1979, 1980; Brasitus and Schachter, 1980,1982) or composition (Bloj and Zilversmit 1982; Forstner et al, 1968; Hauser et al, 1980; Kawai et al, 1974; Millington and Gritchely, 1968; Penzes, 1970; Profirov, 1981) of the brush border membrane (BBM) of the intestine in health or disease. We have recently reported methods to isolate brush border membranes and to analyze their lipid content (Yakymyshyn et al, 1982). This study was undertaken to determine the enzyme marker content and lipid composition of intestinal brush border membrane from weanling, young and mature rabbits.

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METHODS

Studies were performed on weanling (aged 20-22 days and weighing 500 g), young (aged 6 weeks and weighing 2.0-2.5 kg), and mature (aged over 1 year and weighing over 5 kg) female New Zealand rabbits. Weanling animals were weaned on the day of sacrifice. Young and mature rabbits were fed a standard Purina® chow diet until time of sacrifice. Animals were sacrificed by injection of sodium thiopental into the marginal ear vein (1 ml/kg body weight). The proximal jejunum and distal ileum were studied.

The procedures for obtaining mucosa isolation of BBM, marker analyses, lipid analyses, and studying villus morphology are described in detail in Appendices 1-4. All data was expressed as mean ± standard error of the mean. A minimum of six animals were used in each group. Each brush border membrane preparation was obtained from one animal. The unpaired t-test was used to determine the significance of the difference between the mean values of the weanling, young and mature animals.

RESULTS

1. Marker Content of Brush Border Membranes

Various markers of purification have been assayed to determine the purity and the variability of our BBM preparation. Sucrase is an enzyme which is specific to the BBM, and is thought to be a measure of cell maturity. Alkaline phosphatase activity is thought to be a marker of cell immaturity. Na$^+$K$^+$ATPase activity is quite specific for the basolateral membrane, while -glucuronidase is a marker for lysosomal contamination. The presence of DNA represents contamination due to nuclear material. As shown in Table II-1, our BBM preparations are contaminated with very little Na$^+$K$^+$ATPase and -glucuronidase activity, and no detectable levels of nuclear contamination. All markers are expressed as U/g protein.

The mg wet weight/cm length of the jejunum and ileum was higher in both the young and the mature than in the weanling animals ($p<0.05$). The protein content of the jejunal and ileal brush border membranes (BBM) was similar in weanling and mature animals, but was lower in young rabbits (Table II-1). The BBM sucrase activity in young and mature rabbits was increased 3-fold over weanling rabbits in the jejunum, and 2-fold in the ileum. The jejunal and ileal BBM alkaline phosphatase were lower in mature and weanling than in young rabbits ($p<0.05$); no significant difference was noted between weanling and mature animals. The ratio of alkaline phosphatase/sucrase in the jejunum and ileum was similar when young vs weanling animals were compared, but was several-fold higher when comparing weanling with mature or young with mature rabbits. There was no difference between age groups in the BBM Na$^+$K$^+$-ATPase or DNA, but the jejunal BBM B-glucuronidase was lower in the young than in the weanling animals (Table II-1).

The differences noted between the jejunum and the ileum were influenced by the age of the animals (Table II-1). The mg wet weight/cm length was lower in the ileum than in the jejunum for weanling animals, whereas only in the mature rabbits was the BBM protein and sucrase lower in ileum than in jejunum. In contrast, the BBM alkaline phosphatase was lower in ileum than in jejunum for the weanling, young and mature rabbits, but only in weanling animals was the ratio of alkaline phosphatase/sucrase lower in ileum than jejunum.

2. Lipid Content of Brush Border Membranes

In weanling rabbits the BBM content of total phospholipid, free cholesterol and the total phospholipid/cholesterol ratio were significantly lower in the ileum than in the jejunum (Table II-2). In mature animals the BBM content of total and free cholesterol was lower, and the ratio of total phospholipid/total cholesterol was higher in the ileum than in the jejunum ($p<0.05$). In young animals the lipid content was similar in the jejunum and ileum.

With aging, there was an increase in the jejunal BBM content of bile acids, cholesterol and cholesterol esters, when comparing mature vs weanling animals. Only total free fatty acids and cholesterol esters were increased when comparing the jejunum of young vs weanling animals. In the ileum, aging (mature vs wealing) was associated with increased BBM total phospholipid and total phospholipid/total cholesterol. In the jejunum of mature animals, the free cholesterol content was higher, the total free fatty acids content was lower, and the cholesterol ester content of jejunum and ileum was lower than in young animals.

With aging, the percentage of the jejunal BBM lipid content comprised of cholesterol and of free fatty acid rose, while only the free fatty acid component rose in the ileal BBM (Table II-3). Several changes were observed in the phospholipid content with aging (Table II-4). The quantity of the jejunal BBM lecithin first increased (young vs weanling) and then decreased (mature vs young), while the proportion of lecithin was similar in weanling and young animals, and then decreased in the mature rabbits. This resulted in a decrease in the proportion and yet an increase in the quantity of lecithin (mature vs weanling). Both the proportion and the quantity of sphingomyelin first increased (young vs weanling), then decreased (mature vs young) with aging. The increase in the proportion of sphingomyelin was responsible for the decreased proportion of phosphatidyl ethanolamine (young vs weanling). The decreased quantity and proportion of lecithin and sphingomyelin (mature vs young) was explained by the increased quantity and proportion of phosphatidyl ethanolamine (mature vs young) and phosphatidyl serine (mature vs weanling). The quantity and proportion of phosphatidyl inositol remained unchanged in the jejunum of weanling, young and mature rabbits. No lysolecithin was detected in BBM from jejunum or ileum of weanling, young or mature animals.

In the rabbit ileum, the quantity of lecithin, sphingomyelin, and phosphatidyl ethanolamine increased with aging (young vs weanling, mature vs weanling). The proportion of lecithin was similar between weanling and young animals, but decreased between mature and weanling animals. The proportion of sphingomyelin increased (young vs weanling, mature vs weanling), probably due to the decreased proportion of phosphatidyl inositol and lecithin. The phosphatidyl serine content in the ileum, like the jejunum, increased in quantity and proportion between mature and weanling animals, and also between mature and young animals. This increase could be responsible for the decrease in the proportion of lecithin and phosphatidyl inositol in the BBM. The proportion of ileal phosphatidyl inositol decreased with age (young vs weanling, mature vs weanling). The proportion of phosphatidyl ethanolamine remained unchanged with aging.

No differences in phospholipid composition were observed between the jejunum and ileum of weanling animals. In the ileum of young animals, the quantity of lecithin, and the quantity and proportion of phosphatidyl inositol, were decreased as compared to that of the jejunum. In the ileum of mature animals, the quantity of sphingomyelin was increased relative to that of the jejunum.

In the jejunum, the quantity and proportion of choline phospholipid first increased (young vs weanling), and then decreased (mature vs young) with aging. In the ileum, choline phospholipid increased in quantity, but not proportion with aging. The amine phospholipids increased quantitatively in both jejunum and ileum with aging, but only the jejunal amine phospholipids increased in proportion. This resulted in an initial rise and then fall in the jejunal choline phospholipid/amine phospholipid ratio. There were no significant changes in the ileal choline phospholipid/amine phospholipid ratio with aging (Table II-5). In the jejunum of mature rabbits, the BBM choline phospholipids (nmol/mg protein) and the ratio of choline/amine phospholipid were lower but the BBM amine phospholipids were higher ($p<0.05$) than in young animals. In the ileum the choline phospholipid content (nmol/mg protein) was higher in mature than in weanling rabbits ($p<0.05$).

3. Morphology of the Intestinal Mucosa

a) Comparison Between Jejunum and Ileum

The villus height was greater in jejunum than in ileum but this difference was significant only in the young animals (Tables II-6 and

II-7). The crypt depth was lower in the ileum than in the jejunum of mature rabbits. The villus width at half height was less in the ileum than jejunum in the weanling animals. The villus surface area is significantly greater in the jejunum than in the ileum in weanling, young, and mature animals. In the weanling animals, the jejunal villi contained more cells per villus than the ileal villi. In contrast, the number of cells per villus did not differ significantly between the jejunum and ileum of young and mature animals. Cell size did not vary between jejunum and ileum. The density of the villi (no. of villi/mm^2 serosa) was greater in the ileum than in the jejunum of weanling and mature animals. Only in the young animals was the mucosal surface area significantly greater in the jejunum than in the ileum.

b) Comparison Between Animals of Different Ages

A number of morphological measurements were greater in the jejunum and ileum of young as compared to weanling rabbits: height of villus and number of cells per villus, villus surface area, number of villi per mm length, and number of villi per mm^2 serosa. The larger villus height in the young rabbit jejunum resulted in a significant increase in mucosal surface area with aging. Cell size did not change with age. With further aging, villus height decreased but villus thickness increased, so that the villus surface area remained the same when comparing young vs mature animals. With aging, the number of villi per mm serosal length decreased, resulting in a decreased number of villi per mm^2 serosa. This decrease in villus density was associated with a decrease in mucosal surface area of the jejunum but not the ileum, when comparing mature vs young animals. Aging was associated with an increase in the villus surface area for both the jejunum and ileum of rabbits. Aging, as determined from the comparison of weanling, young and mature animals, was associated with an increase, then a decrease in jejunal mucosal surface area. The mucosal surface area of the ileum was not affected by aging.

4. Correlations Between Intestinal Structure, Biochemistry and Transport Function

With aging, the height of the villus was significantly correlated with the villus surface area ($y=-9+0.81x$, $r=0.840$, $p<0.05$), with the mucosal surface area ($y=-0.1+0.03x$, $r=0.885$, $p<0.05$), and with the number of mucosal cells per villus ($y=31+0.31x$, $r=0.967$, $p<0.01$). The BBM sucrase activity was significantly correlated with the villus surface area ($y=-10+0.46x$, $r=0.910$, $p<0.05$). The number of mucosal cells per villus was significantly correlated to villus surface area ($y=-77+2.52x$, $r=0.842$, $p<0.05$), mucosal surface area ($y=-1.6+0.08x$, $r=0.829$, $p<0.05$), and villus height ($y=-65+2.97x$, [illegible]0.968, $p<0.01$). No significant correlation exists between the villus and the mucosal surface area. The ratio of BBM alkaline phosphatase/sucrase showed no correlation with any of the morphological parameters. The maximal transport rate of jejunal glucose uptake (Thomson, 1979) was not correlated with any of the morphological parameters or with the BBM ratio of alkaline phosphatase/sucrase. When the bulk phase was stirred at 600 rpm, the effective resistance of the intestinal unstirred water layer (Thomson, 1980a,b) was significantly correlated with mucosal surface area ($y=-3.4+3.12x$, $r=0.954$, $p<0.05$). When the bulk phase was unstirred and unstirred layer resistance was high, the effective resistance of the intestinal stirred water layer was not correlated with any of the morphological parameters.

DISCUSSION

The weanling animals, as compared with the young or mature rabbits, have lower levels of BBM sucrase and alkaline phosphatase in jejunum and ileum, (Table II-1), resulting in a progressive decline in the value of the ratio alkaline phosphatase/sucrase (AP/S) in the BBM of jejunum and ileum as animals age. Similar findings have been reported by other workers (Hohn et al, 1978; Nordstrom and Dahlqvist, 1972; Suntzeff and Angeletti, 1961; Welsh et al, 1974). The AP/S ratio of the weanling ileum is significantly lower than that of the jejunum, possibly suggesting that the rabbit ileum matures more quickly than the jejunum. The ratio AP/S from cells from the upper third of the villus is lower than the values of AP/S obtained from cells near the villus base. We did not isolate enterocytes from different portions of the villus in animals of various ages. However, the lower value of AP/S associated with aging may indicate that the villi from older animals contain a greater proportion of more mature cells. This possibility is supported by absorption studies which have indicated a greater maximal transport rate for glucose uptake into the jejunum of mature as compared with weanling rabbits (Thomson, 1979).

Aging was associated with increased amounts and/or proportions of BBM free fatty acids, cholesterol (Tables II-2 and II-3) phosphatidyl serine and phosphatidyl ethanolamine (Table II-4). In the jejunum, aging was associated with decreased amounts and/or proportions of lecithin and sphingyomyelin (Table II-4), possibly due to the increased amounts and proportions of phosphatidyl serine and phosphatidyl ethanolamine. Aging was associated with an overall increase in the amount of phospholipids in the ileal BBM. There was an increased proportion of phosphatidyl serine and sphingomyelin in the ileum possibly due to the decreased proportion of lecithin and phosphatidyl inositol. The initial increase in choline phospholipids (weanling vs young) resulted in an increased choline/amine phospholipid ratio. This is followed by a decrease in choline phospholipids and an increase in amine phospholipids (young vs mature) such that the choline phospholipid/amine phospholipid returned to that seen in weanling rabbits. However, aging was not associated with changes in the BBM ratio of total phospholipid/total cholesterol (Table II-2). Aging has a variable influence on the passive absorption of lipids with a decline in the rate of uptake of cholesterol, fatty acids and fatty alcohols (Hollander and Morgan, 1979a,b,; Holt and Dominguez, 1981; Thomson, 1980a; Thomson, 1981). The correlation between these changes in BBM lipid content and membrane permeability is unclear. With aging, the uptake of medium-chain length fatty acids falls, and the relative permeability of the jejunum to fatty acids (as assessed by the measurement of the incremental change in free energy) also falls (Thomson, 1980a). Also, in the rabbit, the uptake of cholesterol and fatty alcohols declines with aging (Thomson, 1981). Clearly these absolute and relative changes in lipid permeability could not be explained by differences in intestinal morphology, since there was no difference in the mucosal surface area when comparing weanling vs mature rabbits (Table II-6 and II-7). However, the fluidity of the BBM was not measured in these studies, and the age-related changes in passive permeability may have been influenced by alterations in the quantity or the ratios of the different phospholipids.

With aging, both the quantity (nmoles/mg protein) and the percentage of the jejunal membrane lipid comprised of cholesterol rose (Tables II-2 and II-3). Also, cholesterol uptake is decreased with aging (Thomson, 1981); thus the specific activity of the test dose of [^{14}C]-cholesterol in the BBM would differ between the different animal groups. If the BBM cholesterol were in the appropriate physico-chemical state, then the greater BBM cholesterol content in the older rabbits might be sufficient to create an unfavorable concentration gradient for the uptake of the test dose of [^{14}C]-labeled cholesterol from the intestinal lumen. This could result in the lower uptake of cholesterol in older as compared with the younger rabbits (Thomson, 1981). In contrast, cholesterol uptake is higher in older than in younger rats (Hollander and Morgan, 1979a). It is not yet established whether this difference is related to species variations in intestinal function, morphology, or in BBM lipid content and/or fluidity.

The uptake of lipids at different sites along the intestine has not been reported in weanling animals, but in mature rabbits cholesterol uptake is similar in the jejunum and in the ileum (Thomson, 1980b). The greater uptake of medium-chain length fatty acids into the jejunum as compared with the ileum appears to be due to a greater functional surface area of the proximal intestine, rather than due to differences in the membrane permeability, measured as the incremental change in free energy. The ratio of total phospholipid/total cholesterol was lower in BBM from the jejunum as compared with the ileum of mature rabbits (Table II-2). Thus the greater uptake of medium-chain length fatty acids into the jejunum as compared with the ileum may also be due to greater permeability properties of the jejunal BBM. This could be related to differences in the lipid content between the proximal and the distal intestinal BBM, to differences in the membrane surface area between the jejunum and ileum of young animals (Table II-6 and II-7), or to differences in the functional surface area between the proximal and distal intestine (Thomson, 1980a). The BBM from ileum of mature animals contained less cholesterol than did BBM from jejunum, and the ratio of phospholipid/ cholesterol was higher in distal as compared with proximal intestine (Table II-2). On this basis, it might have been predicted that cholesterol uptake would be greater into the ileum than into the jejunum; however, this was not the case (Thomson, 1980b). In this study, the lipid content of the BBM was obtained from the tips as well as from the bases of the villi. It is unknown whether the lipid content of the BBM changes along the villus, nor is it known whether all portions of the villus contribute similarly to the uptake of cholesterol. If cholesterol were largely absorbed by cells near the villus tip, then measurements of the lipid content of the BBM obtained from all portions of the villus, such as was performed in this study, would not necessarily reflect the physicochemical events occurring in the absorbing tip cells.

It is controversial whether the mucosal surface area of the intestine changes with aging (Clarke, 1977; Ecknauer et al, 1982; Hohn et al, 1978; Hollander and Morgan, 1979a; Kapadia and Baker, 1976; Pang et al, 1983; Penzes and Skala, 1977; Suntzeff and Angeletti, 1961). Ecknauer et al (1982) demonstrated that with aging rats, there was a slight increase in villus height between 91 and 731 days of age, but the mucosal surface area did not significantly change with age. In this study the villus surface area does increase with age, but the mucosal surface area of the jejunum increases (weanling vs young), then decreases (young vs mature), so that there is no difference between the mucosal surface area of the jejunum of weanling as compared with mature animals (Table II-6). In contrast, Hohn et al (1978) have shown that by light microscopy the mucosa of the jejunum of 30 month old rats becomes atrophied compared to that of four month old rats. Comparable findings have been described by others (Suntzeff and Angeletti, 1961) but neither group calculated the surface area of individual villus or the mucosal surface of a standard intestinal segment. Thus, there are differences with aging in the various parameters describing intestinal morphology, but the effect of aging on the mucosal surface area depends upon the relative ages of the groups being compared.

The measurements made in this study give an estimation of the mucosal surface area based on the villus morphology. It is known the microvilli are responsible for increasing this surface area at least 20 times. Microvillus morphology was not measured here, but clearly the magnitude of the differences in mucosal surface area calculated here are significant. It would be of interest to measure the microvillus morphology so that its impact on the mucosal surface area could be assessed.

While no appreciable difference between four month and 30 month old rats was demonstrable by enzyme histochemistry for alkaline phosphatase, a marked loss in activity of alkaline phosphatase in the proximal intestine of senile rats was shown in disc electropherograms (Hohn et al, 1978). These workers suggested that the villous atrophy observed in their senile rats, and the diminution of the villus epithelium it caused, are manifested in the electropherograms as loss of enzyme activity. In the histochemical preparations, the individual enterocytes showed a largely identical enzyme content and a uniform pattern of intracellular enzyme distribution in both adult and senile rats. This suggested that the enzymatic differentiation of the enterocytes of old animals was not appreciably changed and that the loss of activity in the mucosal homogenates was solely caused by the diminution in the total number of enterocytes with aging. In the present study, the alkaline phosphatase activity per unit protein of the brush border membrane of the jejunum first increased, then declined with age. This suggests that age itself was associated with a decrease in specific activity of this enzyme, independent of any change in the number of enterocytes or the surface area of the membrane. The most significant difference in enzyme activity with age (weanling vs young, or weanling vs mature rabbits) was the increase in sucrase activity per unit protein and the decrease in the ratio of alkaline phosphatase/sucrase (Table II-1). Since sucrase is present in larger amounts as the villus cell matures, this suggests that the older animals have a greater proportion of more mature cells along the villus.

It is unknown what proportion of the villus surface area is involved in the uptake of a given lipid. The uptake of galactose (Kinter and Wilson, 1965) and amino acids (King et al, 1981; Paterson et al, 1980) occurs from the upper third of the villus. No change is observed in the mucosal surface area between the jejunum and the ileum, or between the mature versus the weanling intestine (Table II-6 and II-7). It is unknown whether a similar portion of the villus was involved in lipid uptake in the animals of different ages. Furthermore, the lipid content of all portions of the villus was analyzed together, and there may have been major changes in the compositon of that portion of the membrane through which permeation of the lipids occurs.

The individual fatty acid constituents of the BBM were not determined in this study. Aging is associated with variations in fatty acid composition of erythrocyte and plasma lipids in the rat (Ciccoli and Comporti, 1983). The intestinal microvillus membrane from newborn rabbits contains significantly more phospholipids, cholesterol and glycolipids than adult animals (Pang et al, 1983); there was also a higher lipid-to-protein ratio, but the phospholipid/cholesterol ratios were unchanged. Similarly, in this study BBM cholesterol increased (Tables II-2 and II-3), and the phospholipid/cholesterol ratio was also unaffected by aging for the jejunum, but not for the ileum (Table II-2). Studies using electron spin resonance and spin label methods with 5-doxylstearic acid as a probe have suggested that the microvillus membrane from newborns were more "disordered" and had greater "fluidity" than did microvillus membrane from adult animals. The compositon of a membrane may influence its structure and function (Cullis and Hope, 1980; Pilch et al, 1980; Shattil and Cooper, 1976; Suntzeff and Angeletti, 1961). A more disordered microvillus membrane such as exists in newborn rabbits (Pang et al, 1983) could allow for greater intestinal uptake of macromolecules (Walker et al, 1972), but would not explain the lower uptake of cholesterol or fatty acids (Thomson, 1981). The greater "disordered" nature of the newborn BBM may have been due to its greater content of glycolipid (Pang et al, 1983), or to the differences in the BBM content or proportion of free fatty acids, lecithin, sphingomyelin, phosphatidyl ethanolomine, or phosphatidyl serine (Table II-4).

The weanling animal is in the process of passing through a period of rapid post-natal development which includes histological changes as well as the appearance of brush border membrane enzymes. In addition, the milk diet composition of the weanling animals differs greatly from the chow diet of young and mature animals. These differences were not taken into consideration when comparing weanling animals with young and mature animals. This study was undertaken to compare the differences in intestinal transport, brush border membrane composition, and villus morphology of three stages in the life of a rabbit. Differing diets and growth rates are after all, a part of the maturing process in any species.

It is proposed that aging is associated with a complex interaction of a continuum of morphological, biochemical and functional changes. Some measurements increase from weanling to young animals, and then remain stable; for example, sucrase, free cholesterol, amine phospholipids. Other measurements increase only with aging when comparing young to mature animals: J_d^m for glucose (Thomson, 1979). Other measurements decline when comparing young to mature animals: BBM AP and AP/S, lecithin, sphingomyelin, villus height, the number of cells per villus, villus surface area, and the uptake of fatty acids and cholesterol. Finally, some measurements remain constant throughout the first year of life of the rabbit: size of villus cells, ileal mucosal surface area, and the BBM ratio of total phospholipid/total cholesterol. Thus the aging intestine represents a continuation of a process initiated in youth. Changes in intestinal membrane function are not closely associated with changes in membrane structure. Finally, the signals responsible for the changes in intestinal form and function which occur with aging must now be identified.

Table II-1 ENZYME MARKER CONTENT OF INTESTINAL BRUSH BORDER MEMBRANES OF RABBITS OF DIFFERENT AGES

Membrane Marker	Weanling (6)		Young (8)		Mature (6)	
	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
Mucosal scrapings, mg wet weight/cm length	34 ± 3	25 ± 2*	84 ± 10^{+}	64 ± 6^{+}	61 ± 6^{+}	50 ± 6^{+}
Protein, mg/g wet weight	3.24 ± 0.24	3.73 ± 0.32	2.19 ± 0.23^{+}	2.15 ± 0.42^{+}	4.10 ± 0.36††	3.00 ± 0.28*
Sucrase, U/g protein	382 ± 78	337 ± 59	1079 ± 114^{+}	845 ± 94^{+}	1270 ± 95^{+}	697 ± 42^{+},*
Alkaline phosphatase, U/g protein	606 ± 31	254 ± 67*	1437 ± 234^{+}	766 ± 133^{+}*	699 ± 100††	307 ± 43††,*
Alkaline phosphatase/ sucrase	2.0 ± 0.4	0.8 ± 0.2*	1.5 ± 0.3	0.9 ± 0.2	0.6 ± 0.1^{+},††	0.4 ± 0.1††
$Na^{+}K^{+}$-ATPase, U/g protein	78 ± 21	N/A	63 ± 14	66 ± 17	58 ± 19	N/A
B-glucuronidase, U/g protein	1.43 ± 0.19	0.96 ± 0.14	0.71 ± 0.12^{+}	0.67 ± 0.11	0.98 ± 0.12	0.91 ± 0.13
DNA, mg/g protein	0	0	0	0	0	0

N/A not available
*, $p<0.05$, jejunum vs ileum
+, $p<0.05$ weanling vs young or weanling vs mature
††, $p<0.05$ young vs mature

Table II-2 LIPID CONTENT OF BRUSH BORDER MEMBRANES FROM INTESTINE OF RABBITS OF DIFFERENT AGES

Lipid	Weanling (6)		Young (6)		Mature (6)	
nmol/mg protein	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
Total free fatty acids	52 ± 21	64 ± 14	196 ± 15^{+}	194 ± 16	94 ± 23††	128 ± 40
Total bile acids	0	0.5 ± 0.3	1.7 ± 0.9	0.9 ± 0.7	2.1 ± 0.3+	1.3 ± 0.4
Total phospholipid	280 ± 27	156 ± 24*	374 ± 8	281 ± 48	344 ± 13	366 ± 27+
Total cholesterol	267 ± 10	210 ± 26	304 ± 14	252 ± 20	306 ± 7+	235 ± 21*
free	250 ± 11	187 ± 22*	242 ± 10	201 ± 14	273 ± 5††	213 ± 16*
esters	20 ± 2	23 ± 4	61 ± 6^{+}	52 ± 10^{+}	33 ± 4+††	21 ± 6††
Total phospholipid/ total cholesterol	1.1 ± 0.1	0.6 ± 0.1*	1.2 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	1.6 ± 0.1+*

*, $p<0.05$, Jejunum vs Ileum

+, $p<0.05$, weanling vs young, or weanling vs mature

††, $p<0.05$, mature vs young

Table II-3 EFFECT OF AGING ON PERCENT LIPID COMPOSITION OF RABBIT INTESTINAL BRUSH BORDER MEMBRANE

Lipid	Weanling		Young		Mature	
%	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
Cholesterol	53.2±10.0	69.0±3.7	55.6±3.6	56.6±5.5	66.6±1.1††	66.6±1.8
Cholesterol ester[@]	0	0	0	0	0	0
Free fatty acid	0.06±0.04	0.14±0.13	1.8±1.0	2.1±0.5^{+}	0.50±0.09^{+}	0.14±0.06††,*
Triglyceride	1.1±0.3	0*	0	0	1.3±0.8	0
Diglyceride	0	0	0	0	0	0
Monoglyceride, phospholipid, bile acid	35.9±1.4	30.9±3.8	42.5±4.5	41.4±5.7	31.7±1.6	33.3±1.8

[@] cholesterol ester levels are not detected by HPTLC. The enzymatic determinations of total and free cholesterol are much more sensitive: cholesterol ester levels can be determined.

*, $p<0.05$, jejunum vs ileum
+, $p<0.05$, weanling vs young, or weanling vs mature
††, $p<0.05$, young vs mature

Table II-4 EFFECT OF AGING ON PHOSPHOLIPID COMPOSITION OF RABBIT INTESTINAL BRUSH BORDER MEMBRANE

Phospholipid Composition	Weanling		Young		Mature	
	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
Lecithin,						
nmol/mg protein	103±17	67±3	155±8^{+}	108±12+,*	111±5††	111±6+
%	40.2±2.5	42.8±1.8	41.6±1.9	39.2±2.5	33.3±1.0+,††	31.3±1.9+
Lysolecithin,						
nmol/mg protein	0	0	0	0	0	0
%	0	0	0	0	0	0
Sphingomyelin,						
nmol/mg protein	20±4	15±1	64±4+	51±9+	42±2+,††	58±3+,*
%	7.8±0.8	9.4±0.6	17.2±1.2+	18.0±1.1+	12.6±0.4+,††	16.7±2.1+
Phosphatidyl serine,						
nmol/mg protein	11±2	7±1	16±6	13±5	24±3+	49±10+,††
%	4.5±0.4	4.3±0.5	4.2±1.7	4.2±1.4	7.2±0.9+	13.1±2.0+,††,*
Phosphatidyl ethanolamine,						
nmol/mg protein	107±24	57±4	109±7	95±14+	134±3††	129±20+
%	40.2±3.2	36.3±2.8	29.2±1.8^{+}	33.4±1.7	40.1±0.7††	34.5±3.5
Phosphatidyl inositol,						
nmol/mg protein	19±3	11±1	29±3	15±2*	23±1	16±3
%	7.3±0.5	7.2±0.6	7.8±1.0	5.2±0.3+,*	6.8±0.2	4.4±0.6^{+},*

+, $p<0.05$, weanling vs young, or weanling vs mature
*, $p<0.05$, jejunum vs ileum
††, $p<0.05$, young vs mature

Table II-5 EFFECT OF AGING ON CHOLINE AND AMINE PHOSPHOLIPID COMPOSITION OF RABBIT INTESTINAL BRUSH BORDER MEMBRANE

Phospholipid	Weanling		Young		Mature	
	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
Choline phospholipid						
nmol/mg protein	123±20	82±4	219±10+	159±21+	153±6††	169±8+
%	48.0±3.1	52.2±2.3	58.8±2.6	57.2±2.5	45.8±0.7††	48.0±4.0
Amine phospholipid						
nmol/mg protein	118±25	63±4	125±12	107±17	158±4††	177±25+
%	44.7±3.4	40.6±2.8	33.5±3.2	37.6±2.7	47.4±0.7††	47.6±3.5
Choline/amine phospholipid	1.1±0.2	1.3±0.2	1.8±0.2+	1.6±0.2	1.0±0.03††	1.1±0.2

*, $p<0.05$, Jejunum vs ileum
+, $p<0.05$, weanling vs young or weanling vs mature
††, $p<0.05$, young vs mature

Table II-6 EFFECT OF AGING ON RABBIT JEJUNAL MORPHOLOGY

Morphological Parameter	Weanling	Young	Mature
Crypt depth, µm	52 ± 6	40 ± 5	66 ± 5††
Villus:			
height, µm	343 ± 54	759 ± 80+	481 ± 40††
width @$^1/_2$height, µm	121 ± 8	120 ± 9	123 ± 4
bottom width, µm	122 ± 12	92 ± 3+	140 ± 8††
thickness, µm	257 ± 10	250 ± 6	385 ± 14+††
surface area, μm^2/villus	240 ± 15	604 ± 68+	529 ± 41+
No. of cells per villus	135 ± 16	266 ± 27+	174 ± 11††
Cell size, µm	5.1 ± 0.8	5.7 ± 0.6	5.5 ± 0.5
No. of villi/ mm serosal length[a]	8.5 ± 0.8	11.0 ± 0.4+	7.2 ± 0.4††
No. of villi/ mm serosal length[b]	3.7 ± 0.4	4.0 ± 0.1	2.6 ± 0.1+††
No. of villi/ mm^2 serosa	31.6 ± 2.9	44.2 ± 1.5+	18.8 ± 1.0+††
Mucosal surface area mm^2/mm^2 serosa	9.1 ± 1.4	25.6 ± 3.0+	10.2 ± 1.2††

+, $p<0.05$, weanling vs young, weanling vs mature

††, $p<0.05$, mature vs young

Table II-7 EFFECT OF AGING ON RABBIT ILEAL MORPHOLOGY

Morphological Parameter	Weanling	Young	Mature
Crypt depth, µm	41 ± 4	$59 \pm 6^{+*}$	$44 \pm 4^{*}$
Villus:			
height, µm	243 ± 24	$508 \pm 23^{+*}$	$260 \pm 25^{+\dagger\dagger}$
width @$^1/_2$height, µm	$76 \pm 2^{*}$	$106 \pm 6^{+}$	$100 \pm 10^{+}$
bottom width, µm	93 ± 7	$143 \pm 8^{+*}$	120 ± 13
thickness, µm	$107 \pm 3^{*}$	$172 \pm 3^{+*}$	$314 \pm 11^{+\dagger\dagger*}$
surface area, µm^2/villus	$95 \pm 9^{*}$	$297 \pm 16^{+*}$	$302 \pm 56^{+*}$
No. of cells per villus	$86 \pm 7^{*}$	$205 \pm 17^{+}$	$136 \pm 16^{+\dagger\dagger}$
Cell size, µm	5.6 ± 0.5	5.0 ± 0.2	4.9 ± 1.0
No. of villi/ mm serosal length^a	11.1 ± 08	$7.2 \pm 0.4^{+*}$	8.7 ± 0.9
No. ov villi/ mm serosal length^b	$9.2 \pm 0.4^{*}$	$5.9 \pm 0.1^{+*}$	$3.2 \pm 0.1^{+\dagger\dagger*}$
No. of villi/mm^2 serosa	$102.1 \pm 7.4^{*}$	$41.4 \pm 2.2^{+}$	$27.7 \pm 2.9^{+\dagger\dagger*}$
Mucosal surface area, mm^2/mm^2 serosa	10.1 ± 1.6	$12.1 \pm 0.8^{*}$	8.7 ± 2.4

$+$ $p<0.05$, weanling vs young, weanling vs mature

$\dagger\dagger$ $p<0.05$, mature vs young

$*$ $p<0.05$, jejunum vs ileum

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CHAPTER III

EFFECT OF FASTING AND DIABETES MELLITUS ON RAT INTESTINAL BRUSH BORDER MEMBRANE MARKER ENZYMES LIPID COMPOSITION AND VILLUS MORPHOLOGY[1]

INTRODUCTION

Diabetes mellitus is associated with increased passive intestinal uptake of fatty acids and cholesterol, suggesting that there are changes in the passive permeability properties of the intestine (Thomson, 1980; Thomson, 1983a). Enhanced uptake of actively transported solutes such as glucose may be due to a greater number of carriers in the diabetic intestine (Thomson, 1981). There may be minor morphological and biochemical changes in the diabetic intestine, such as higher villi and increased sucrase levels (Nakabou et al., 1980; Gourley et al, 1983; Miller et al, 1977; Younoszai and Schedl, 1972). Changes in lipid uptake in the diabetic rat are influenced by dietary modification (Thomson and Rajotte, 1983a, 1983b) and by insulin (Thomson, 1984), but the mechanism of the change in the permeability properties of the intestinal brush border membrane (BBM) has not been delineated. Changes in membrane lipid composition may be associated with changes in membrane function (Boggs, 1980; Chapman, 1975; Keelan et al, 1984). Alterations in membrane fluidity are associated with changes in membrane function in some tissues (Brasitus et al, 1979; Gourley et al, 1983; Kamada and Otsuji, 1983; Pilch et al, 1980) but there is only limited information on the fluidity of the BBM (Brasitus et al, 1979; Gourley et al, 1983). The lipid content of the BBM has been reported for non-diabetic rats (Chapelle and Guilles-Baillien, 1983; Forstner et al, 1968; Gourley et al, 1983) and 5-day diabetic rats (Gourley et al, 1983). The present study was undertaken to determine the lipid composition of the intestinal brush border membrane in fasting and fed, 14-day diabetic rats and non-diabetic control rats.

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METHODS

Studies were performed on female Wistar rats weighing 200-220 g. Drug-induced glucose intolerance was produced in the first group by intravenous administration of the cell cytotoxic agent, streptozotocin (80 mg/kg). A second group served as a non-diabetic control group and was subjected to intravenous administration of bacteriostatic saline. The induction of glucose intolerance was assessed 3-4 days post-streptozotocin injection by the presence of hyperglycemia and glucosuria using the glucose oxidase method. All diabetic animals had blood glucose values of greater than 300 mg/dl. Control animals did not exhibt hyperglycemia or glucosuria. The control and 14-day post-streptozotocin diabetic rats were studied after a 15 hour fast, or were allowed *ad libitum* access to standard Purine® chow until the time of sacrifice. Animals were sacrificed by intraperitoneal injection of ketamine (1 ml/300 g body weight). Only the proximal jejunum was studied.

The procedures for obtaining the mucosa, isolation of the BBM, marker analyses, lipid analyses, and studying villus morphology are described in detail in Appendices 1 - 4.

All results are expressed as mean ± standard error of the mean. Six animals were used in each group. Each brush border membrane preparation was obtained from one animal. The significance of the difference between mean values was determined by the unpaired t-test.

RESULTS

1. Marker Analysis of Brush Border Membranes of Control and Diabetic Rats.

In control and diabetic rats, the ratio, mg intestinal wet weight/cm length of intestine, was lower ($p<0.05$) in fasting than in fed animals (Table III-1). Fasting had no significant effect on the ratio mg protein/g wet weight of intestine. Fasting did increase the amount of protein in diabetic animals, when expressed as mg/cm length. When activity was expressed as U/g protein, there were no significant differences between fed versus fasting or between control versus diabetic rat brush border membrane (BBM) sucrase, alkaline phosphatase or -glucuronidase. Sucrase and alkaline phosphatase activities were similar in diabetic as compared with control rats when activity was expressed as U/cm length of intestine. Feeding had no effect on sucrase and alkaline phosphatase activities in the control rats. Feeding had no effect on the alkaline phosphatase activity in diabetic rats (Table III-1), but did result in an increase in sucrase activity when expressed as U/cm length.

2. Lipid Composition of Brush Border Membranes of Control and Diabetic Rats.

In the control rats, the effect of fasting was associated with significantly higher levels of brush border membrane (BBM) total phospholipids, cholesterol esters and total phospholipid/total cholesterol (Tables III-2,and III-3), both when the lipid content was expressed as nmoles/mg protein (Table III-2), or nmoles/cm length of intestine (Table III-3). In the diabetic rats, the effect of fasting was associated with significantly higher levels of BBM total cholesterol and free cholesterol but lower total free fatty acids, when expressed as nmol/mg protein (Table III-2). However when the results were expressed as nmol/cm length, only the decline in BBM total free fatty acids achieved statistical significance (Table III-3). When comparing diabetic with control rats, there was higher BBM total phospholipid, higher total phospholipid/total cholesterol in fed diabetic animals and higher free cholesterol in fasting diabetic animals, when the results were expressed as nmol/mg protein (Table III-2). When the results were expressed as nmol/cm length, only the BBM total phospholipid was higher in fed diabetic than control rats (Table III-3).

The percent lipid composition of the BBM is shown in Table III-4. In control rats, feeding had no effect on BBM % lipid composition. In diabetic rats, fasting was associated only with an increase in the % lipid composition of phospholipid-monoglyceride-bile acid, cholesterol and cholesterol ester ($p<0.05$). When comparing diabetic with control rats, the only difference in the % lipid composition was a significantly lower content of BBM cholesterol esters in fasting than fed diabetic rats.

The individual phospholipids of the BBM were measured. There were no significant differences in the BBM phospholipid composition between fed and fasting control and between fed and fasting diabetic rats, or between diabetic versus control animals when expressed as a % of total phospholipid (Table III-5). When the BBM phospholipid content was expressed as nmol/mg protein (Table III-6), fasting was associated with a significant increase in BBM lecithin and phosphatidyl ethanolamine in control rats. The BBM lecithin and phosphatidyl ethanolamine was higher in fed diabetic than in fed control animals ($p<0.05$).

Fasting had no effect on the BBM choline or amine phospholipids (Table III-7) in the diabetic rats. In control rats, fasting was associated with significantly increased BBM choline phospholipids (nmol/mg protein) as well as amine phospholipids (nmol/mg protein). The BBM of fed diabetic rats had a significantly greater content (nmol/mg protein) of choline and amine phospholipids than control animals. Fasting diabetic animals demonstrated no differences in BBM choline or amine phospholipid content when compared to fed diabetic and fasting control animals.

3. Morphology of Intestine

In the jejunum of control rats (Table III-8), fasting was associated with lower villus height, higher villus bottom width, lower villus surface area, lower number of villi per mm^2 serosa, and lower mucosal surface area ($p<0.05$). In the jejunum of diabetic rats, fasting was associated with lower villus bottom width, and higher number of villi per mm serosal $length_a$ ($p<0.05$). The only morphological difference between the jejunum of fed control and diabetic rats was the higher number of cells per villus in diabetic animals ($p<0.05$). Several morphological differences exist between the jejunum of fasting diabetic and control rats: the diabetic animals had a greater villus height, villus surface area, number of cells per villus, number of villi per mm serosal $length_a$, number of villi per mm^2 serosa, and mucosal surface area ($p<0.05$).

Microvillus morphology was examined in fed control and diabetic rats and found to be similar between the two groups (Table III-8). Microvillus surface area was not included in the calculation of mucosal surface area in this study. Although the microvilli increased the mucosal surface area approximately 25 times (Palay and Karlin, 1959), this study indicates that a change in villus height also has a profound effect on mucosal surface area. Microvilli were not examined in the fasted animals, but previous studies with hamster jejunum indicate that microvilli are 25-30% taller in fed than fasted animals (Misch et al, 1980).

There was a direct linear relationship between the height of the villus and several morphological parameters in fed and fasted control and diabetic animals. None of the morphological parameters measured showed a significant correlation with sucrase activity or the alkaline phosphatase/sucrase ratio. The villus height was significantly correlated with the villus surface area ($y=-27+1.22X$, $r=0.962$ $p<0.01$), mucosal surface area ($y=2.7+0.02x$, $r=0.859$, $p<0.01$), and the number of cells per villus ($y=49+0.25x$, $r=0.839$, $p<0.01$). The villus surface area is significantly correlated with the mucosal surface area ($y=4.0+0.01x$, $r=0.778$, $p<0.05$), and the number of cells per villus ($y=56+0.20x$, $r=0.864$, $p<0.01$). The number of cells per villus is significantly correlated with the mucosal surface area ($y=1.2+0.06x$, $r=0.798$, $p<0.05$).

DISCUSSION

Several workers have reported significantly increased sucrase activities in the intestinal mucosal homogenates (Caspary et al, 1974; Riecken et al, 1979; Tandon et al, 1975) and brush border membranes

(Cerda et al, 1972; Tandon et al, 1978) of diabetic patients. We were unable to confirm previous reports of significantly increased alkaline phosphatase activity (Caspary et al, 1972) and significantly increased sucrase activity (Caspary et al, 1972; Gourley et al, 1983; Olsen and Rogers, 1970, 1971; Schedl et al, 1983; Younoszai and Schedl, 1972) in the intestinal BBM of diabetic rats (Table 1). In this study the diabetic animals' hyperglycemia was comparable to that reported by Miller et al (1977). Our data fails to show a significant effect of overnight fasting on any of the marker enzymes. Possible differences in food intake are unlikely to be the only cause of enhanced enzyme activities, since pair-feeding experiments between control and diabetic animals have shown that the functional changes associated with diabetes are maintained despite the absence of increased mucosal mass (Nakabou et al., 1974; Olsen and Rogers, 1970). In the adult rat, 3-day fasting decreases specific and total disaccharidase activities (Blair et al., 1963; Deren et al., 1967), which are restored by carbohydrate feeding. Elevated specific and total activities of sucrase in the mucosal homogenates of streptozotocin diabetic rats has been demonstrated (Schedl et al, 1983; Yamada et al, 1980). These workers suggested that this elevation is a response to the diabetic state and is independent of enteral factors such as luminal nutrition, bile or pancreatic secretions. However, not all workers have demonstrated increases in the BBM sucrase activities between control and diabetic animals (Yamada et al., 1980) or between control and diabetic patients (Chaudhary and Olsen, 1973). Pothier and Hugan (1982) reported increases in diabetic mouse intestinal sucrase and alkaline phosphatase activities, both when examined in homogenates and in BBM. Although total activities of all brush border membrane enzymes were at least doubled in diabetic mice, their specific activities remained relatively unchanged, due to the increase in the total protein content in the BBM. Thus technical differences and the manner of expressing the results likely explain the apparent discrepancy in data between the studies.

Cholesterol and medium-chain length fatty acid uptake is increased in fed diabetic rats as compared with fed control animals, but these differences disappeared with fasting (Thomson, 1980). With fasting the only differences between the diabetic and control rats was in the increased free cholesterol (Table III-2) and in the % of cholesterol esters in the BBM (Table III-4). These changes were possibly of major physiological significance, since cholesterol uptake was similar in the fasting diabetic and fasting non-diabetic control rats (Thomson, 1980). Cholesterol content influences the fluidity of membranes and perhaps also the permeability of membranes (Demel and DeKruyff, 1976). The increased free cholesterol content of fasted diabetic jeunal BBM may be involved in the mechanism which appears to reverse the cholesterol permeability changes observed in the jejunum of fed diabetic rats. In contrast, in fed rats, there were numerous differences in the BBM content of lipids in the diabetic as compared with control animals: in the fed diabetic animals there were increases in the BBM total phospholipid, the ratio of total phospholipid/total cholesterol (Tables III-2 and III-3), increased lecithin and phosphatidyl ethanolamine (Table III-6), resulting in increased choline and amine phospholipids (Table III-7).

The increased BBM total phospholipid in the diabetic fed animals may have altered the "fluidity" properties of the membrane, thereby altering permeability and favouring enhanced uptake. Changes in membrane function may be associated with changes in membrane fluidity (Brasitus et al., 1979; Demel and DeKruyff, 1976; Gourley et al, 1983; Pilch et al, 1980). The lipid fluidity of the brush border membrane is low compared to other mammalian membranes (Brasitus et al, 1980; Gourley et al, 1983; Nassar et al, 1981). The lipid transition influences the function of certain of the microvillus membrane proteins (Brasitus et al., 1979): Arrhenius plots of D-glucose transport, for example, show a discontinuity in slope near the lipid transition temperature, whereas the microvillus membrane digestive enzymes such as sucrase yield one slope on an Arrhenius plot and may be classified as "extrinsic" activities, functionally independent of the lipid transition. The increased phospholipid content of the BBM of the fed diabetic animals would be anticipated to be associated with an increased membrane fluidity. This increased fluidity in turn might be expected to be associated with increased intestinal permeability to cholesterol or other lipophilic probes. Furthermore, the change in the lipid composition of the BBM in diabetic animals might be predicted to be associated with changes in "intrinsic" functions whereas there would be no changes in "extrinsic" function. Indeed, glucose uptake is altered in diabetic rats (Thomson, 1981), but in our hands sucrase activity is unchanged (Table III-1).

Diabetes mellitus extensively alters lipid metabolism in several tissues (Faas and Carter, 1981,1983; Whiting et al., 1977; Woods et al., 1981), and the lipid composition of a variety of tissues is altered in streptozotocin-diabetic rats (Chandramouli and Carter, 1975; Clark et al, 1983; Faas and Carter, 1981, 1983; Holman et al., 1983; Lemieux et al, 1984). In renal tissues of diabetic rats, no consistent change was noted in the total phospholipid content nor in the proportion of various phospholipids (Clark et al., 1983). However, diabetic animals showed a consistent reduction of arachidonic acid content in phosphatidyl choline and phosphatidyl ethanolamine in whole renal cortex, plasma membranes purified from renal cortex, and in isolated glomeruli. Associated with the fall in arachidonic acid (20:4) was a rise in linoleic acid (18:2) in the samples studied. Insulin returned the fatty acid composition to normal. These changes in diabetic kidney fatty acid composition in phospholipids are similar to changes previously observed in diabetic liver (Faas and Carter, 1981, 1983). The expected tendency of the shift toward more saturated, shorter-chain fatty acids in diabetic kidney would be to create a more rigid or less fluid membrane. That these biochemical changes may have functional significance is suggested by these workers' demonstration of alterations in the temperature-dependence of adenylate cyclase in renal plasma membranes of diabetic animals.

Furthermore, Holman et al (1983) demonstrated that linoleic and dihomo- -linolenic acids were increased in phospholipids from most tissues in the diabetic rat, whereas arachidonic acid was decreased in most tissues. It is possible that these changes in the fatty acid compositions of phospholipids in diabetes may be influenced by diet, since mammalian cells require exogenous precursors of the long-chain polyunsaturated fatty acids (Holman et al., 1982). Linoleic and linolenic acid are the precursors of some of these polyunsaturated fatty acids, and the arachidonic acid deficiency described by Holman and co-workers may have effects on cellular functions in diabetes.

The previously reported studies of Gourley et al (1983) revealed only a decrease in fatty acids of the neutral lipid fraction of the microvillus membrane from fasting diabetic animals. Although there was an increase in the membrane total phospholipids and in the ratio of phospholipids/cholesterol, these differences failed to achieve statistical significance. These workers also noted that diabetes does not alter the enterocyte brush border membrane sufficiently to affect DPH fluorescence polarization determinations. However, there were several important methodological differences between the studies of Gourley et al and our study: their animals were younger, had been diabetic for a shorter period, and were fasting. In our study, the major biochemical differences between the brush border membranes of diabetic and control animals were observed when the animals were fed (Tables III-2 and III-3). Thus the functional differences in intestinal transport reported in the fed diabetic rat are associated with changes in the lipid composition of the brush border membrane.

The only significant jejunal morphological difference between control and diabetic rats when the animals were fed was the greater number of cells per villus (Table III-8). There were no significant differences in BBM sucrase or alkaline phosphatase activities (Table III-1) under these conditions. Thus the increased lipid uptake in the fed diabetic animals is unlikely related to altered intestinal morphology. Other workers have also suggested that changes in intestinal mass and absorptive function are not linked, but rather appear to be under independent control (Richter et al., 1983).

Fasting influences numerous aspects of villus morphology (Table III-8), but only in the control animal was there a significant decrease in mucosal surface area. These shorter villi of lower surface area in the control animals were associated with changes in the lipid content of the BBM (Tables III-2 - III-7). These changes in BBM lipids were unlikely due to altered maturity of the cells, since there were no changes in the depth of the crypts (Table III-3), or the BBM sucrase or alkaline phosphatase activities (Table III-2). Indeed, it is uncertain whether these morphological and lipid changes were due to the presence of food in the intestinal tract or to hormone changes, since many of these alterations did not occur in the diabetic animals. Some workers have suggested that the changes in mucosal morphology observed with diabetes is due to hyperphagia (Riecken et al, 1979). In this study, the morphology of fed and fasted diabetic jejunum was similar. This suggests that the morphological changes observed were due to the diabetes not hyperphagia. There is little information on the effect of fasting on the uptake of lipids. The uptake of cholesterol is similar in fasting diabetic and control animals, and only in fed diabetic rats is the uptake of cholesterol increased (Thomson, 1980). Thus, fasting removes the differences in cholesterol uptake by the diabetic rats, but the mechanism for this effect remains unclear. The lowering of the cholesterol uptake in fasted diabetic rats is unlikely to be explained by alterations in villus morphology (Table III-8).

Fasting had a quantitatively different effect on the BBM lipids of diabetic rats as compared to control rats. Total free fatty acids, total cholesterol and free cholesterol were increased in the fasted diabetic rats, whereas total phospholipid (lecithin, phosphatidyl ethanolamine), and total phospholipid/cholesterol ratio were increased in the fasted control rats.

The net result is an increase in neutral lipids with fasting in the diabetic rats, and an increase in total phospholipids with fasting in the control rats. The difference in lipid composition between the fasting diabetic and control rats does not affect cholesterol uptake (Thomson, 1980). In the fed animals, the BBM in the jejunum of diabetic rats is composed of a higher total phospholipid content than the control rats. Since very little change is observed in villus morphology, the increase in phospholipid composition may be involved in the enhanced cholesterol uptake observed in fed diabetic rats (Thomson, 1980).

TABLE III-1 EFFECT OF FASTING AND DIABETES ON JEJUNAL BRUSH BORDER MEMBRANE MARKERS

Membrane Marker	Control Rats		Diabetic Rats	
	Fed (n=5)	Fasting (n=6)	Fed (n=6)	Fasting (n=6)
Mucosal scrapings, mg wet wt/cm length	29 ± 2	22 ± 1*	41 ± 3†	23 ± 2*
Protein, mg/g wet wt	2.11 ± 0.26	2.28 ± 0.19	1.55 ± 0.22	1.63 ± 0.28
mg/cm length	0.063 ± 0.010	0.049 ± 0.005	0.061 ± 0.010	0.035 ± 0.006*
Sucrase, U/g protein	1153 ± 116	1099 ± 86	1462 ± 102	1168 ± 188
U/cm length	0.079 ± 0.009	0.054 ± 0.006	0.093 ± 0.018	0.042 ± 0.007*
Alkaline phosphatase, U/g protein	1875 ± 525	1516 ± 545	1653 ± 352	1450 ± 308
U/cm length	0.131 ± 0.028	0.077 ± 0.027	0.108 ± 0.032	0.051 ± 0.014
Alkaline phosphatase/sucrase	1.68 ± 0.39	1.37 ± 0.46	1.18 ± 0.27	1.55 ± 0.46
B-glucuronidase, U/g protein	0.337 ± 0.035	0.284 ± 0.044	0.325 ± 0.057	0.429 ± 0.066
DNA, μg/mg protein	0	0	0	0

* p <0.05, fed versus fasting

† p < 0.05, control versus diabetic

TABLE III-2 EFFECT OF FASTING AND DIABETES ON JEJUNAL BRUSH BORDER MEMBRANE LIPIDS (nmol/mg protein)

Lipid nmol/mg protein	Control Rats		Diabetic Rats	
	Fed (n=5)	Fasting (n=6)	Fed (n=6)	Fasting (n=6)
Total free fatty acids	262 ± 59	140 ± 27	329 ± 64	149 ± 13*
Total bile acids	1.3 ± 1.1	0.2 ± 0.2	1.8 ± 1.1	0.6 ± 0.5
Total phospholipids	235 ± 20	556 ± 60*	465 ± 33†	512 ± 40
Cholesterol total	255 ± 13	298 ± 20	266 ± 27	350 ± 17*
free	249 ± 12	236 ± 10	253 ± 23	324 ± 15†,*
esters	6 ± 4	44 ± 12*	14 ± 5	26 ± 6
Total phospholipid/ total cholesterol	0.9 ± 0.1	2.0 ± 0.3*	1.8 ± 0.2†	1.5 ± 0.1

* $p<0.05$, fasting versus fed

† $p<0.05$, control versus diabetic.

Table III-3 EFFECT OF FASTING AND DIABETES ON JEJUNAL BRUSH BORDER MEMBRANE LIPIDS (nmol/cm serosal length)

Lipid nmol/cm serosal length		Control Rats		Diabetic Rats	
		Fed (n=5)	Fasting (n=6)	Fed (n=6)	Fasting (n=6)
Total Free Fatty Acids		18.7 ± 6.4	6.8 ± 1.4	19.8 ± 4.8	5.1 ± 0.6*
Total Bile Acids		0.12 ± 0.10	0.01 ± 0.01	0.12 ± 0.07	0.03 ± 0.02
Total Phospholipids		20.8 ± 3.2	46.9 ± 7.0*	43.2 ± 3.4†	42.2 ± 5.1
Cholesterol	total	22.2 ± 1.4	24.3 ± 1.4	25.2 ± 3.4	29.0 ± 2.8
	free	21.8 ± 1.5	20.4 ± 0.9	24.0 ± 3.1	26.8 ± 2.3
	esters	0.4 ± 0.3	3.8 ± 1.1*	3.4 ± 2.0	1.9 ± 0.5

* $p<0.05$, fasting vs fed

† $p<0.05$, control vs diabetic

TABLE III-4 EFFECT OF FASTING AND DIABETES ON JEJUNAL BRUSH BORDER MEMBRANE LIPIDS (% OF TOTAL LIPID)

Lipid %	Control		Diabetic	
	Fed	Fasting	Fed	Fasting
Phospholipid Monoglyceride Bile acid	34.3 ± 1.6	35.1 ± 1.8	32.6 ± 1.8	40.0 ± 1.8*
Diglyceride	1.7 ± 0.5	2.4 ± 0.5	1.8 ± 0.6	3.0 ± 0.7
Cholesterol	31.7 ± 2.0	38.7 ± 2.1	30.2 ± 1.0	37.9 ± 1.1*
Cholesterol Esters	ND	ND	1.6 ± 1.4	ND
Free Fatty Acids	26.9 ± 3.1	20.6 ± 1.8	32.6 ± 2.2	14.9 ± 1.5*†
Triglyceride	5.4 ± 2.1	3.2 ± 0.4	1.2 ± 0.6	4.2 ± 1.5

* $p<0.05$, fed versus fasting

† $p<0.05$, control versus diabetic

ND none detected

TABLE III-5 . EFFECT OF FASTING AND DIABETES ON JEJUNAL BRUSH BORDER MEMBRANE PHOSPHOLIPIDS (% OF TOTAL PHOSPHOLIPIDS)

Phospholipid %	Control		Diabetic	
	Fed	Fasting	Fed	Fasting
Lysolecithin	ND	ND	ND	ND
Sphingomyelin	11.2 ± 3.1	8.6 ± 0.8	15.8 ± 3.3	12.0 ± 3.0
Lecithin	41.7 ± 4.0	43.4 ± 1.9	38.2 ± 3.5	36.2 ± 2.5
Phosphatidyl Serine	3.1 ± 1.2	1.8 ± 0.6	0.8 ± 0.7	2.2 ± 1.2
Phosphatidyl Inositol	9.2 ± 2.5	7.5 ± 0.6	6.4 ± 1.4	4.4 ± 1.7
Phosphatidic Acid	1.7 ± 1.5	0.9 ± 0.8	2.6 ± 1.5	7.9 ± 4.6
Phosphatidyl Ethanolamine	33.2 ± 4.0	37.8 ± 3.0	36.2 ± 3.0	37.4 ± 3.6

t-testing failed to show a significant difference in the phospholipid composition between fed versus fasting groups, or between diabetic versus control rats.

ND, none detected

TABLE III-6 EFFECT OF FASTING AND DIABETES ON JEJUNAL BRUSH BORDER MEMBRANE PHOSPHOLIPIDS (nmol/mg protein)

Phospholipid nmol/mg protein	Control		Diabetic	
	Fed	Fasting	Fed	Fasting
Lysolecithin	ND	ND	ND	ND
Sphingomyelin	29 ± 11	46 ± 5	58 ± 9	44 ± 7
Lecithin	101 ± 17	238 ± 28*	172 ± 8†	193 ± 12
Phosphatidyl Serine	8 ± 3	10 ± 6	4 ± 3	12 ± 6
Phosphatidyl Inositol	20 ± 6	43 ± 35	30 ± 8	21 ± 9
Phosphatidic Acid	3 ± 3	6 ± 6	12 ± 8	22 ± 19
Phosphatidyl Ethanolamine	74 ± 6	210 ± 35*	174 ± 25†	199 ± 20

* $p<0.05$, fed versus fasting

† $p<0.05$, control versus diabetic

ND none detected

TABLE III-7 EFFECT OF FASTING AND DIABETES ON JEJUNAL BRUSH BORDER MEMBRANE CHOLINE AND AMINE PHOSPHOLIPID COMPOSITION

Phospholipid	Control		Diabetic	
	Fed	Fasting	Fed	Fasting
Choline Phospholipid				
%	52.9 ± 6.6	52.0 ± 2.6	54.0 ± 4.1	48.2 ± 3.7
nmol/mg protein	129 ± 27	284 ± 33*	245 ± 15†	256 ± 21
Amine Phospholipid				
%	36.2 ± 3.4	39.6 ± 2.5	37.0 ± 2.9	41.3 ± 2.9
nmol/mg protein	82 ± 5	221 ± 34*	178 ± 25†	219 ± 16
Choline/Amine Phospholipid	1.65 ± 0.42	1.37 ± 0.16	1.56 ± 0.21	1.22 ± 0.16

* $p<0.05$, fed versus fasting

† $p<0.05$, control versus diabetic

TABLE III-8 EFFECT OF FASTING AND DIABETES ON JEJUNAL MORPHOLOGY

Morphological Parameter	Control		Diabetic	
	Fed	Fasting	Fed	Fasting
Crypt depth, µm	121±9	98±16	114±4	118±7
Villus				
height, µm	407±19	326±14*	454±28	519±52^{+}
width at $^{1}/_{2}$height,µm	161±13	176±19	159±8	134±12
bottom width, µm	144±13	175±15*	153±14	111±8*
thickness µm	356±36	329±22	367±32	349±26
surface area µm^{2}/villus	487±28	390±27*	537±29	561±63^{+}
Number of cells per villus	130±8	119±6	172±10^{+}	187±14^{+}
Villus cell size,µm	6.3 ± 0.3	5.5 ± 0.2	5.3 ± 0.3	5.6±0.6
Number of villi per mm serosal length$_{a}$	7.4±0.6	6.1±0.6	6.9±0.7	9.5±0.7*,$^{+}$
Number of villi per mm serosal length$_{b}$	3.1±0.4	2.8±0.3	3.2±0.3	2.8±0.2
Number of villi per mm^{2} serosa	23.0±2.0	16.7±1.6*	21.8±2.2	26.3±2.1†
Mucosal surface area, mm^{2}/mm^{2} serosa	11.4±1.2	6.4±0.5*	11.8±1.5	14.0±1.7^{+}
Microvillus height, µm	1.62 ± 0.03	ND	1.40 ± 0.03	ND
Number of microvilli per µm	6.7 ± 0.1	ND	7.3 ± 0.3	ND

* $p<0.05$, Fed versus Fasting

$^{+}$ $p<0.05$, Control versus Diabetic

ND, not done

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CHAPTER IV

EFFECT OF CHRONIC ETHANOL CONSUMPTION AND FOOD DEPRIVATION ON RABBIT INTESTINAL VILLUS MORPHOLOGY AND BRUSH BORDER MEMBRANE CONTENT OF LIPID AND MARKER ENZYMES[1]

INTRODUCTION

Acute and chronic ethanol feeding in the rabbit is associated with changes in the passive permeability to fatty acids and cholesterol (Thomson 1984a;Thomson 1984b) and with alterations in intestinal lipid metabolism (Baraona et al, 1975). The effect of ethanol on membrane fluidity is controversial. Tillotson et al (1981) have suggested that acute exposure of the intestine to ethanol is not associated with changes in the fluidity of the brush border membrane (BBM), whereas Hunter and co-workers (1983) have suggested that ethanol does have a significant fluidizing effect on the BBM. Changes in the lipid content of membranes may be associated with changes in their membrane fluidity (Chin et al, 1978), and these changes in membrane fluidity also affect enzyme function (Wilsom and Hoyumpa, 1979). Acute exposure to ethanol modifies the activity of several intestinal brush border membrane enzymes (Dinda et al, 1979; Dinda and Beck, 1982). The lipid content of BBM vesicles obtained from animals with acute exposure to ethanol has been reported (Mansbach 1983), but the lipid content of the BBM in chronic ethanol-fed animals has not been studied and the effect of chronic ethanol exposure on BBM enzymes has also not been reported. Accordingly, this study was undertaken to determine the effect of chronic ethanol feeding and food deprivation on the enzyme and lipid content of the jejunal and ileal BBM.

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METHODS

Adult female New Zealand rabbits weighing 2 - 2.5 kg were raised for 6 weeks on a standard Purina® chow, with one group allowed *ad libitum* access to chow and 15% ethanol (V/V) added to their drinking water (EF); a second group received *ad libitum* access to chow (control, *ad libitum*, CAL); a third group was fed with sufficient chow to match the weight gain of the ethanol-fed rabbits (control, weight gain, CWG); and a fourth group was fed the same amount of chow as the ethanol-fed rabbits (control, food restricted, CFR). Animals were sacrificed by anaesthetic overdose injection of sodium thiopentol into the marginal ear vein (1 ml/kg body weight). Both proximal jejunum and distal ileum were studied.

The procedures for obtaining the mucosa, isolation of the BBM marker analyses, lipid analyses, and morphology were described in detail in Appendices 1 - 4.

Data were derived from 7-11 animals in each group. Each brush border mebrane preparation was obtained from one animal. All data were expressed as mean ± standard error of the mean. The unpaired t-test was used to determine the significance of the difference between the mean values of the groups.

RESULTS

1. Marker Content of the Brush Border Membranes of Rabbit Jejunum and Ileum

The marker content of the brush border membranes (BBM) of the jejunum was not significantly different from that of the ileum of control-ad libitum and control-food restricted animals (Tables IV-1 and IV-2). The jejunal BBM sucrase(S) and alkaline phosphatase (AP) activity was significantly higher than in the ileal BBM of control-weight gain animals.

The BBM marker content of the control-food restricted animals was similar to the control-ad libitum animals, except for a slight decrease in the ileal BBM protein when expressed as mg/g wet weight. The BBM marker content of the control-weight gain animals was similar to the control-ad libitum animals, except for a significantly increased sucrase activity in the jejunal BBM.

The BBM marker content of the ethanol-fed animals was similar between the jejunum and ileum except for a greater sucrase activity and lower -glucuronidase activity in the jejunal than in the ileal BBM.

Nordstrom et al (1968) demonstrated that AP activity increases progressively from crypt to villus tip while S activity is maximal over the mid portion of the villus. The AP/S ratio varies along the villus, such that a decreased ratio suggests a greater proportion of relatively mature cells. Upon comparison with the control-ad libirum and control-food restricted animals, the S activity was higher while the AP activity and AP/S were lower in the jejunal BBM of ethanol-fed animals. Upon comparison with the control-weight gain animals, only AP activity and AP/S were lower in the jejunal BBM of ethanol-fed animals.

The only difference between ethanol-fed rabbits and the three control groups in ileal BBM marker content was the protein content. The ileal BBM protein content from ethanol-fed animals was higher than in the BBM from control-ad lib animals when expressed as mg protein/g wet weight ($p<0.05$), but there was no difference when the protein content was expressed as mg protein/cm ileal length. The protein content of ileal BBM from ethanol-fed animals was higher than BBM of control-food restricted animals when expressed either as mg protein/g wet weight or mg protein/cm length. When comparing the ileal BBM of ethanol-fed animals with control-weight gain animals, the protein content was greater in the ethanol-fed animals only when expressed as mg protein/cm length.

There were no differences between the ethanol-fed animals and any of the three control groups in the Na^+K^+ATPase activity, -glucuronidase activity, or DNA content of the jejunal and ileal BBM. This indicates a similar degree of membrane purification among all groups.

2. Lipid Content of Brush Border Membranes

In the control ad-lib animals, there were no significant differences between jejunum and ileum in the total bile acids, total phospholipids, cholesterol esters and the ratio of total phospholipid/total cholesterol (PL/C), but total free fatty acids, total cholesterol and free cholesterol were lower in the ileum than the jejunum (Tables IV-3 and IV-4). There was no difference in the jejunal and ileal BBM lipid composition between the three control groups (Table IV-3 and IV-4). Although the jejunal BBM total phospholipid content was similar, the cholesterol content (total, free, esters) was significantly higher than in the ileal BBM of ethanol-fed animals. This resulted in a lower PL/C ratio in the jejunal BBM than in the ileal BBM. In addition, the ileal BBM content of total bile acids was greater than in the jeunal BBM of ethanol-fed animals. There was a significant decrease in the total free fatty acid content in the jejunal BBM of the ethanol-fed rabbits, as compared to the control rabbits ($p<0.05$). In the ethanol-fed animals, jejunal BBM phospholipid content tended to be lower, resulting in a significant decrease in PL/C, as compared with control-ad lib and control-weight gain animals. The total bile acid content of the ileal BBM was higher in the ethanol-fed animals than in the control-ad libitum and control-weight gain animals ($p<0.05$).

The total phospholipid content was higher in BBM of jejunum than ileum of control-ad lib animals (Tables IV-3 and IV-4), possibly due to the qualitative and quantitative increase in lecithin and the quantitative increase in phosphatidyl ethanolamine in the jejunum (Table IV-5). The total phospholipid content of BBM was similar in the jejunum and ileum of ethanol-fed animals (Tables IV-3 and IV-4). Accordingly, the quantitative and percent composition of phospholipids was also similar (Table IV-5). The jejunal BBM phospholipid composition content of BBM of jejunum was similar in ethanol-fed and in control-ad lib rabbits (Table IV-5), except for a quantitative decrease in lecithin in the ethanol-fed rabbits. The phospholipid composition was similar in the ileal BBM of control-ad lib and ethanol-fed animals (Table IV-5).

The percent choline PL and amine PL composition was similar in the jejunum and ileum of control-ad lib animals (Table IV-6). Quantitatively, the choline PL and amine PL composition were both lower in the ileum than the jejunum. This could be explained by the quantitative decrease in lecithin and phosphatidyl ethanolamine in the ileal BBM. The choline/amine PL ratios were similar in the jejunum and ileum of control-ad lib animals.

In the ethanol-fed animals, the choline PL, amine PL, and choline/amine PL ratio were qualitatively and quantitiatively similar in the jejunum and ileum. Although the proportion of choline PL was similar (expressed as percent), the quantitative amount of choline PL (expressed as nmol/mg protein) was lower in the jejunum of ethanol-fed than the control-ad lib animals. In contrast, the proportion of amine PL was higher in the jejunum of ethanol-fed than control-ad lib animals, but the quantitative amount of amine PL was similar. The choline/amine PL ratio was lower in the jejunum of ethanol-fed than in control-ad lib animals. This decreased ratio could be explained by the quantitative decrease in choline PL or by the qualitative increase in amine PL. The quantitative decrease in choline PL may be explained by the combined effect of the slight (but not statistically significant) quantitative decreases in lysolecithin and sphingomyelin, as well as the significant quantitative decrease in lecithin in the jejunal BBM of the ethanol-fed rabbits as compared to the control-ad lib rabbits. The qualitative increase in amine PL may be explained by the combined effect of the slight (but not statistically significant) increase in the percent phosphatidyl serine and phosphatidyl ethanolamine in the jejunum of ethanol-fed rabbits, as compared to the control-ad lib animals. The qualitative (percent) and quantitative (nmol/mg protein) amounts of choline PL, amine PL, and the choline/amine PL ratio were similar in the ileum of ethanol-fed and control-ad lib animals.

3. Intestinal Morphological Characteristics

a. Comparison Between Jejunum and Ileum

Tables IV-7 and IV-8 illustrate the effect of ethanol-feeding and food deprivation on intestinal morphology.

In the control-ad lib animals, villus height and villus thickness were decreased in the ileum, resulting in a decreased villus surface area and mucosal surface area.

In the control-food restricted animals, villus height, villus width at 1/2 height, villus thickness, and villus surface area were lower in the ileum than the jejunum. The jejunal villus cell size was also increased. The number of villi per mm^2 serosa was significantly lower in the jejunum than in the ileum. This resulted in a similar mucosal surface area for the jejunum and ileum.

In the control-weight gain animals, villus thickness was significantly decreased, producing a decreased villus surface area. The number of villi per mm^2 serosa was significantly lower in the jejunum than in the ileum. The resulting mucosal surface area was similar for the jejunum and ileum.

In the ethanol-fed animals, villus height, villus surface area and mucosal surface area were two times greater in the jejunum than in the ileum. Accordingly, the number of cells per villus and the villus cell size were also significantly greater in the jejunum than in the ileum.

b. Comparison Between Different Animals Groups

Jejunal villus height was similar among control groups, but alterations in villus thickness resulted in a larger villus surface area in control-food restricted animals than control-ad lib and control-weight gain animals. The food deprived animals had a significantly lower jejunal villus density which resulted in a lower mucosal surface area as compared to the control-ad lib animals. The number of cells per villus and the cell size were similar among control groups, with the exception of the lower number of cells per villus in the control-weight gain animals. Crypt depth was similar among control groups. In ethanol-fed animals, jejunal villus height and villus surface area were significantly larger than control-ad lib and control-weight gain animals, but were similar to control-food restricted animals. Alterations in jejunal villus density (number of villi per mm^2 serosa) of ethanol-fed animals resulted in a mucosal surface area which was similar to control-ad lib animals but was larger than in food-deprived animals. There were more cells per villus in the jejunal villi of ethanol-fed animals than in control animals, but villus cell size and crypt depth were similar.

Ileal villus height was similar among control groups, but variations in villus thickness resulted in a higher villus surface area and mucosal surface area in food-deprived animals as compared to control-ad lib animals. The number of cells per villus was similar among control-ad lib and control-food restricted animals, but was lower in control-weight gain animals. The cell size of control-ad lib animals was smaller than in control-weight gain animals but was larger than in control-food restricted animals. Although villus height in the ethanol-fed animals was similar to the control animals, villus thickness was larger and resulted in a larger villus surface area than in control-ad lib animals. Further variations in villus density did not alter mucosal surface area when comparing the ileum of ethanol-fed animals to control-ad lib and control-weight gain animals. The ileal mucosal surface area was significantly lower in ethanol-fed animals as compared to control-food restricted animals. There were significantly fewer cells in the ileum of ethanol-fed animals than control-food restricted animals. Ileal villus cell size in ethanol-fed animals were similar to control-ad lib animals, were larger than in control-food restricted animals, and were smaller than in control-weight gain animals. Ileal crypt depth was similar in ethanol-fed and in control animals.

c. Morphological Correlations

There was a direct linear relationship between the height of the villus and the villus surface area ($y=-177\pm1.04x$, $r=0.911$, $p<0.01$), and between the height of the villus and mucosal surface area ($y=2.33+0.02x$, $r=0.804$, $p<0.05$). The number of cells per villus was not strongly related to the villus height. Sucrase activity was not significantly correlated with any of the morphological parameters measured.

DISCUSSION

A gradient of function along the intestine has been recognized for many years. As reported by others (Chin and Goldstein, 1977), this study demonstrated lower alkaline phosphatase activity in the ileum than in the jejunum of control-ad lib animals (Tables IV-1 and IV-2). Although the total uptake of fatty acids is greater in the jejunum than in the ileum (Thomson, 1980), the incremental change in free energy is similar for the proximal and the distal small intestine, and similar quantities of cholesterol are absorbed from each site. Although the cholesterol content of the BBM was consistently lower in the ileum than the jejunum of control animals, the PL/C ratio was similar at both sites

(Tables IV-3 and IV-4). Thus the similar permeability properties of the jejunum and ileum towards cholesterol were also associated with similar PL/C ratio in the BBM.

Changes in food intake were associated with minor changes in the lipid content and enzyme activity of the jejunum (Tables IV-1 and IV-3) and ileum (Tables IV-2 and IV-4) in the control groups: sucrase activity was higher in the BBM from the jejunum of control-weight gain than in control-ad lib and control-food restricted animals ($p<0.05$). Protein was lower in BBM from the ileum of control-food restricted than control-ad lib animals only when expressed as mg protein/g wet weight ($p<0.05$). Food deprivation did not have a significant effect on the lipid composition of the BBM. In this study no consistent pattern developed from the analyses of marker enzymes or lipids which correlated with the changes in membrane function in the food-deprived animals.

Ethanol ingestion had a greater effect on the BBM marker and lipid composition of the jejunum than the ileum. The jejunal BBM of ethanol-fed rabbits contained less alkaline phosphatase activity, more sucrase activity, and therefore a lower alkaline phosphatase/sucrase ratio than the control-ad lib rabbits (Table IV-1). The marker content of the ileal BBM was unaffected by ethanol ingestion (Table IV-2). Examination of the jejunal lipid composition revealed a decrease in the total phospholipid content of ethanol-fed animals which resulted in a significantly lowered phospholipid/cholesterol ratio (Table IV-3). The smaller amount of phospholipid present in the jejunal BBM of ethanol-fed animals may be explained by the quantitative decrease in lecithin, a choline phospholipid (Table IV-3). The decreased amount of choline phospholipid results in a lower choline phospholipid/amine phospholipid ratio in the jejunal BBM of ethanol-fed animals (Table IV-6). This altered phospholipid composition may affect membrane fluidity and permeability. Although the total bile acid composition is higher in the ileal BBM of ethanol-fed animals than in the control-ad lib animals, none of the remaining lipids and phospholipids were affected by ethanol-feeding, and therefore could not explain any changes in membrane function observed (Tables IV-4 - IV-6).

In contrast, the reduced jejunal uptake of cholesterol and long chain fatty acids in the 6-week ethanol-fed animals (Raul et al, 1982) was associated with a reduction in the total phospholipid content and PL/C in the jejunum of ethanol-fed as compared with control-ad lib animals (Table IV-3), with a concomitant significant decrease in the amount of lecithin, but without a concomitant change in the pattern of percent phospholipids (Table IV-5). We speculate that the previously-reported jejunal changes in cholesterol and fatty acid uptake in chronic ethanol-fed rabbits (Thomson, 1984b) are related to the changes in the membrane phospholipid composition or phospholipid/cholesterol ratio of the BBM (Tables IV-3, IV-5, IV-6). Morphological changes associated with ethanol feeding, eg. increased villus height and villus surface area, yet unchanged mucosal surface area, do not appear to be related to the altered uptake of lipids in the jejunum.

An optimal range of membrane fluidity appears to be important for many biological functions, and there may be physiological regulatory mechanisms to control membrane fluidity. Hunter and co-workers (1983) reported that membrane fluidity measured by electron paramagnetic resonance showed that acute exposure of the rat intestine to ethanol had a significant fluidizing effect. Membranes from mice that had been subjected to long-term ethanol treatment were relatively resistant to the fluidizing effect of ethanol, suggesting that the membranes themselves had adapted to ethanol (Chin and Goldstein, 1977). Cellular tolerance to the continued presence of ethanol is thought to be due to a change in the physical properties of the lipid bilayer (Chin and Goldstein, 1977; Hill and Bangham, 1975) which, in turn, is presumably due to alternations in membrane lipid composition (Chin et al, 1978; Sun and Sun, 1978). In general, long-term exposure to ethanol results in an increase in fatty acid saturations with arachidonic/linoleic acid ratios being decreased in both mitochondrial and whole liver preparations (Miceli and Ferrell, 1973; Thompson and Reitz, 1978). In addition, increases in liver cholesterol and total phospholipids, ie. phosphatidylcholine have been reported (Fallon et al, 1969; Lefevre et al, 1972; Mendenhall et al, 1969).

The effect of ethanol on the liver is influenced by the dietary content of fat. Although the fatty acid composition of phospholipids of isolated hepatocytes were essentially unaffected by ethanol given to rats on a low-fat (5%) liquid diet, when the dietary fat content was increased to 34%, ethanol significantly decreased the proportion of arachidonate in all the phospholipids examined (Smith et al, 1982). Using the same diets, others have reported similar findings in total mitochondrial fatty acids (Thompson and Reitz, 1978), but not all workers have demonstrated a change in the arachidonate/linoleate ratios of rat liver mitochondiral preparations at liver mitochondrial preparations in animals chronically fed a high-fat diet supplemented with alcohol (Waring et al, 1978). Altering the fat content of ethanol liquid diets was observed to be associated with a two-fold increase in the cholesterol content of isolated hepatocytes (Smith et al, 1982). Previously, there have been no published reports of the effect of chronic ethanol feeding on the lipid content of the intestinal BBM, or of the effect which the level of dietary fat plays in determining the effects of chronic ethanol comsumption on BBM function, morphology or composition.

The effect of ethanol on membranes may in part explain the observed effects of alcohol on intestinal passive permeation, carrier-mediated transport, the activity of membrane-bound enzymes such as lactase, sucrase, maltase, and alkaline phosphatase. Several studies have shown that alcohol inhibits Na^+ K^+ ATPase activity, possibly by its effect on the fluid lipid environment of this membrane-bound enzyme (Wilson and Hoyumpa, 1979), which in turn is of key importance in a number of active transport mechanisms influenced by ethanol, including sugar, amino acids, water, electrolytes, and thiamine. The observed impairment of thiamine movement out of the enterocyte produced by alcohol correlates with a fall in Na^+ K^+ ATPase activity and, as for glucose and amino acid transport, ethanol-induced changes in membrane fluidity have been implicated (Hoyumpa, 1980).

In recent years it has been increasingly reported that a great variety of functional and morphological changes of the upper gastrointestinal tract may occur in alcohol abuse (Wilson and Hoyumpa, 1979). Chronic ethanol feeding increased many morphological parameters in the jejunum including villus height, villus surface area, number of cells/villus, number of villi/mm^2 serosa and mucosal surface area (Table IV-7); thereby providing a possible mechanism for increasing nutrient absorption. This was not the case. Raul et al (1982) demonstrated a reduction in the jejunal uptake of cholesterol and long chain fatty acids in chronic ethanol-fed animals. Chronic ethanol feeding had a much less dramatic effect on the morphology of the ileum. Although villus thickness and villus surface area were increased, the number of villi/mm^2 serosa were decreased such that the mucosal surface area was unchanged (Table IV-8). Food deprivation had a variable effect on mucosal morphology. Although the jejunal mucosal surface area was decreased in control-food restricted animals, it was unaffected by food deprivation in control-weight gain animals. Both villus and mucosal surface areas were increased in the ileum of control-food restricted and control-weight gain animals (Table IV-8).

Acute alcohol ingestion in human volunteers causes a reversable decrease in the activity of intestinal glycolytic enzymes (Greene et al, 1974). Conflicting results concerning the effect of alcohol ingestion on disaccharidase activities in the intestinal mucosa have been reported (Baraona et al, 1974; Madzarovova-Nobejlova, 1971; Perlow et al, 1977). Chronic alcohol consumption also influences intestinal uptake of glucose, fatty acids and cholesterol (Thomson, 1984b). In chronic beer-drinking alcoholics, the mean activity of lactase, maltase, and sucrase in small bowel biopsies was comparable with controls (Bode et al, 1982). Other workers have reported that chronic alcohol feeding is associated with decreased mucosal lactase and alkaline phosphatase activity (Baraona et al, 1974; Hufnagel et al, 1983). On the other hand, Raul et al (1982) noted that feeding a 15% solution of ethanol to rats for 4 weeks provoked stimulation of disaccharidase activities in the proximal jejunum. Perhaps nutritional and species differences contribute to those contradictory findings.

TABLE IV-1 MARKER CONTENT OF JEJUNAL BRUSH BORDER MEMBRANES IN ETHANOL-FED AND FOOD-DEPRIVED RABBITS

Membrane Marker	Control-Ad Libitum (11)	Control-Food Restricted (8)	Control-Weight Gain (10)	Ethanol-Fed (10)
Mucosal scrapings, Mg wet weight/ cm length	78±8	88±7	75±8	75±5
Protein,				
mg/g wet weight	2.40±0.25	1.76±0.27	2.40±0.30	2.44±0.19
mg/cm length	0.19±0.03	0.16±0.03	0.17±02	0.17±0.02
Sucrase, U/g protein	1015±69	962±70	1251±101$^{*,+,++}$	1314±60$^{*,+,++}$
Alkaline phosphatase U/g protein	1092±200	1044±134	1145±90*	816±63$^{+,+++}$
Alkaline phosphatase/ sucrase	1.37±0.21	1.13±0.15	0.89±0.08	0.63±0.06$^{+,++,+++}$
Na^+K^+ATPase, U/g protein	132±42	80±15	83±15	87±18
β-glucuronidase, U/g protein	0.67±0.09	0.76±0.07	0.95±0.16	0.58±0.04*
DNA, mg/g protein	0	0	0	0

* $p<0.05$, jejunum (Table 2) versus ileum (Table 3)

+, $p<0.05$, control-ad libitum versus ethanol-fed, control-ad libitum versus control-food restricted, control-ad libitum versus control-weight gain

++, $p<0.05$, control-food restricted versus ethanol-fed, control-food restricted versus control-weight gain

+++, $p<0.05$, control-weight gain versus ethanol-fed

The numbers given in brackets indicate the number of animals in each experimental group.

TABLE IV-2 MARKER CONTENT OF ILEAL BRUSH BORDER MEMBRANES IN ETHANOL-FED AND FOOD-DEPRIVED RABBITS

Membrane Marker	Control- Ad Libitum (9)	Control- Food Restricted (7)	Control- Weight Gain (10)	Ethanol Fed (10)
Mucosal scrapings, mg wet weight/ cm length	69 ± 7	69 ± 6	65 ± 8	68 ± 3
Protein,				
mg/g wet weight	2.08 ± 0.38	1.73 ± 0.21+	2.32 ± 0.27	3.15 ± 0.28+,++
mg/cm length	0.15 ± 0.03	0.11 ± 0.02	0.14 ± 0.01	0.22 ± 0.03++,+++
Sucrase, U/g protein	907 ± 102	767 ± 26	881 ± 38*	902 ± 111*
Alkaline phosphatase, U/g protein	766 ± 126	787 ± 62	685 ± 100*	609 ± 91
Alkaline phosphatase/ sucrase	0.94 ± 0.15	1.04 ± 0.10	0.81 ± 0.14	0.63 ± 0.11
Na^+K^+ATPase, U/g protein	89 ± 26	142 ± 40	133 ± 26	94 ± 17
β-glucuronidase, U/g protein	0.81 ± 0.17	0.74 ± 0.12	1.21 ± 0.19	0.91 ± 0.08*
DNA, mg/g protein	0	0	0	0

* $p<0.05$, jejunum (Table 2) versus ileum (Table 3)

+ $p<0.05$, control-ad libitum versus ethanol-fed, control-ad libitum versus control-food restricted, control-ad-libitum vs control-weight gain

++ $p<0.05$, control food-restricted versus ethanol-fed, control-food restricted versus control-weight gain

+++ $p<0.05$, control-weight gain versus ethanol-fed

TABLE IV-3 LIPID CONTENT OF BRUSH BORDER MEMBRANES FROM JEJUNUM OF ETHANOL-FED AND FOOD-DEPRIVED RABBITS

Lipid nmoles/mg protein	Control- Ad-Libitum (11)	Control- Food Restricted (8)	Control- Weight Gain (11)	Ethanol Fed (11)
Total free fatty acids	227 ± 23*	192 ± 25*	252 ± 45	112 ± 19+,++,+++
Total bile acids	2.0 ± 0.7	3.8 ± 1.7	1.2 ± 0.5	1.4 ± 0.5*
Total phospholipids	374 ± 8	407 ± 35	441 ± 38	291 ± 44
Total cholesterol	247 ± 11*	289 ± 27*	288 ± 26*	266 ± 38*
free	201 ± 9 *	222 ± 21*	227 ± 21*	217 ± 31*
esters	46 ± 2	67 ± 6*	59 ± 5*	49 ± 7*
Total phospholipid/ total cholesterol	1.52 ± 0.08	1.45 ± 0.14	1.52 ± 0.06	1.10 ± 0.09*,+,+++

* $p<0.05$, jejunum (Table 5) versus ileum (Table 6)

+ $p<0.05$, control ad-libitum versus ethanol-fed, control-ad libitum versus control-food restricted, control ad-libitum versus control-weight gain

++ $p<0.05$, control-food restricted versus ethanol-fed, control-food restricted versus control-weight gain

+++ $p<0.05$, control-weight gain versus ethanol-fed

TABLE IV-4 LIPID CONTENT OF BRUSH BORDER MEMBRANES FROM ILEUM OF ETHANOL-FED AND FOOD-DEPRIVED RABBITS

Lipid nmoles/mg protein	Control-Ad-Libitum (8)	Control-Food Restricted (11)	Control-Weight Gain (10)	Ethanol-Fed (10)
Total free fatty acids	34 ± 20*	52 ± 24*	102 ± 51	47 ± 21
Total bile acids	1.2 ± 0.7	3.8 ± 1.3	1.3 ± 0.5	3.9 ± 1.5*,+,+++
Total phospholipids	281 ± 48	295 ± 37	311 ± 48	274 ± 20
Total cholesterol	164 ± 16*	193 ± 10*	201 ± 16*	168 ± 6*
free	128 ± 12*	154 ± 8*	159 ± 13*	138 ± 5*
esters	36 ± 4	40 ± 2*	41 ± 3*	30 ± 1*
Total phospholipid/ total cholesterol	1.70 ± 0.20	1.50 ± 0.13	1.52 ± 0.15	1.65 ± 0.13*

* $p<0.05$, jejunum (Table 5) versus ileum (Table 6)

+ $p<0.05$, control-ad libitum versus ethanol-fed, control-ad libitum versus control-food restricted, control-ad libitum versus control-weight gain

++ $p<0.05$, control-food restricted versus ethanol-fed, control-food restricted versus control-weight gain

+++ $p<0.05$, control-weight gain versus ethanol-fed

TABLE IV-5 PHOSPHOLIPID COMPOSITION OF BRUSH BORDER MEMBRANES FROM THE JEJUNUM AND ILEUM OF ETHANOL-FED—AND CONTROL RABBITS

Phospholipid Composition	JEJUNUM		ILEUM	
	Control-ad Libitum (7)	Ethanol-Fed (7)	Control-ad Libitum (7)	Ethanol-Fed (7)
Lysolecithin, nmol/mg protein	24 ± 6	13 ± 4	19 ± 4	15 ± 3
%	6.4 ± 1.5	4.4 ± 1.4	6.8 ± 1.4	5.3 ± 1.2
Sphingomyelin, nmol/mg protein	39 ± 4	31 ± 2	35 ± 2	30 ± 2
%	10.5 ± 1.1	10.5 ± 0.7	12.5 ± 0.8	11.0 ± 0.9
Lecithin, nmol/mg protein	124 ± 9	80 ± 9+	70 ± 4*	62 ± 6
%	33.1 ± 2.5	27.6 ± 2.9	24.8 ± 1.3*	22.7 ± 2.0
Phosphatidyl serine, nmol/mg protein	42 ± 8	51 ± 6	38 ± 6	47 ± 4
%	11.2 ± 2.1	17.6 ± 1.9	13.6 ± 2.0	17.2 ± 1.6
Lysophosphatidyl ethanolamine nmol/mg protein	42 ± 6	35 ± 8	37 ± 6	43 ± 6
%	11.2 ± 1.5	11.9 ± 2.9	13.2 ± 2.0	15.8 ± 2.3
Phosphatidyl ethanolamine nmol/mg protein	75 ± 9	68 ± 9	43 ± 5*	47 ± 8
%	20.1 ± 2.3	23.4 ± 3.1	15.2 ± 1.7	17.0 ± 2.8
Phosphatidyl inositol, nmol/mg protein	59 ± 2	61 ± 16	50 ± 3	45 ± 2
%	15.7 ± 0.6	21.0 ± 5.4	17.9 ± 1.1	16.3 ± 0.9
Phosphatidic acid, nmol/mg protein	7 ± 2	7 ± 1	9 ± 2	7 ± 1
%	2.0 ± 0.4	2.4 ± 0.2	3.1 ± 0.8	2.6 ± 0.3

*, $p<0.05$, jejunum versus ileum

+ $p<0.05$, control-ad libitum versus ethanol-fed

TABLE IV-6 EFFECT OF ETHANOL ON CHOLINE AND AMINE PHOSPHOLIPID COMPOSITION OF BRUSH BORDER MEMBRANES FROM THE JEJUNUM AND ILEUM OF ETHANOL-FED AND CONTROL RABBITS

Phospholipid	JEJUNUM		ILEUM	
	Control-Ad Libitum (7)	Ethanol-Fed (7)	Control-Ad Libitum (7)	Ethanol-Fed (7)
Choline phospholipid				
nmol/mg protein	181 ± 12	123 ± 8[+]	119 ± 5[*]	106 ± 4
%	48.3 ± 3.3	42.2 ± 2.7	42.4 ± 1.8	38.7 ± 1.5
Amine phospholipid				
nmol/mg protein	116 ± 3	119 ± 11	80 ± 11[*]	93 ± 10
%	31.1 ± 0.8	41.0 ± 3.7[+]	28.7 ± 3.7	34.0 ± 3.5
Choline/amine phospholipid	1.55 ± 0.10	1.08 ± 0.10[+]	1.79 ± 0.36	1.26 ± 0.14

*, $p<0.05$, jejunum versus ileum

+, $p<0.05$, control-ad libitum versus ethanol-fed

TABLE IV-7 EFFECT OF ETHANOL FEEDING AND FOOD DEPRIVATION ON RABBIT JEJUNAL MORPHOLOGY

Morphological Parameter	Control-Ad Libitum	Control-Food Restricted	Control-Weight Gain	Ethanol-Fed
Crypt depth, µm	40±5	47±4	57±3	55±4
Villus:				
height, µm	759±80	837±97	604±74	1045±39$^{+,+++}$
width @1/2 height, µm	120±9	110±7	98±10	108±4
bottom width, µm	92±3	116±5^{+}	107±12	106±7
thickness, µm	250±6	392±19^{+}	302±12$^{+,++}$	266±18^{++}
surface area, µm^2/villus	604±68	881±98^{+}	487±61^{++}	808±24$^{+,+++}$
No. of cells/villus	266±27	231±20	188±16^{+}	316±18$^{++,+++}$
Villus cell size, µm	5.7±0.6	6.5±0.8	6.4±0.7	6.5±0.3
No. of villi/mm serosal length$_a$	11.0±0.4	8.8±0.4^{+}	8.8±0.7^{+}	9.4±0.5^{+}
No. of villi/mm serosal length$_b$	4.0±0.1	2.1±0.3^{+}	3.6±0.3^{++}	3.9±0.2^{++}
No. of villi/mm^2 serosa	44.2±1.5	18.2±0.8^{+}	31.4±2.6$^{+,++}$	35.2±2.0$^{+,++}$
Mucosal surface area mm^2/mm^2 serosa	25.8±3.0	18.2±2.8	15.8±1.6^{+}	27.8±1.5$^{++,+++}$

*, $p<0.05$, jejunum versus ileum

+, $p<0.05$, control-ad libitum versus ethanol-fed, control-ad libitum versus food-restricted, control-ad libitum versus control-weight gain

++, $p<0.05$, control-food restricted versus ethanol-fed, control-food restricted versus control-weight gain

+++, $p<0.05$, control-weight gain versus ethanol-fed

TABLE IV-8 EFFECT OF ETHANOL FEEDING AND FOOD DEPRIVATION ON RABBIT ILEAL MORPHOLOGY

Morphological Parameter	Control-Ad Libitum	Control-Food Restricted	Control-Weight Gain	Ethanol Fed
Crypt depth, μm	59 ± 6	$37 \pm 3^{+}$	46 ± 4	46 ± 5
Villus:				
height, μm	$508 \pm 23^{*}$	$553 \pm 20^{*}$	571 ± 20	$490 \pm 37^{*}$
width at $^{1}/_{2}$height, μm	106 ± 6	$88 \pm 4^{*+}$	$87 \pm 4^{+}$	99 ± 5
bottom width, μm	$143 \pm 8^{*}$	$98 \pm 7^{+}$	$109 \pm 7^{+}$	$112 \pm 8^{+}$
thickness, μm	$172 \pm 3^{*}$	$228 \pm 11^{*+}$	$200 \pm 8^{*+}$	$269 \pm 12^{+,++,+++}$
surface area, μm^{2}/villus	$297 \pm 16^{*}$	$369 \pm 17^{*+}$	$345 \pm 14^{*+}$	$383 \pm 24^{*+}$
No. of cells/villus	205 ± 17	280 ± 8	$180 \pm 17^{++}$	$199 \pm 15^{*,++}$
Villus cell size, μm	5.0 ± 0.2	$4.0 \pm 0.2^{*+}$	$6.3 \pm 0.2^{+,++}$	$4.7 \pm 0.3^{*,++,+++}$
No. of villi/mm serosal length_{a}	$7.2 \pm 0.4^{*}$	$10.1 \pm 0.6^{+}$	$8.9 \pm 0.4^{+}$	$8.9 \pm 0.5^{+}$
No. of villi/mm serosal length_{b}	$5.9 \pm 0.1^{*}$	$4.5 \pm 0.2^{*,+}$	$5.1 \pm 0.3^{*,+}$	$3.8 \pm 0.2^{+,++,+++}$
No. of villi/mm^{2} serosa	41.4 ± 2.2	$45.5 \pm 2.7^{*}$	$45.9 \pm 2.2^{*}$	$32.9 \pm 1.9^{+,++,+++}$
Mucosal surface area mm^{2}/mm^{2} serosa	$12.1 \pm 0.8^{*}$	$13.0 \pm 1.0^{*,++}$	$16.7 \pm 1.2^{+}$	$15.9 \pm 0.9^{+}$

* $p<0.05$, jejunum versus ileum

+ $p<0.05$, control-ad libitum versus ethanol-fed, control-ad libitum versus food-restricted, control-ad libitum versus control-weight gain.

++ $p<0.05$, control-food restricted versus ethanol-fed, control-food restricted versus control-weight gain.

+++ $p<0.05$, control-weight gain versus ethanol-fed.

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CHAPTER V

EFFECT OF EXTERNAL ABDOMINAL IRRADIATION ON INTESTINAL MORPHOLOGY AND BRUSH BORDER MEMBRANE ENZYME AND LIPID COMPOSITION[1]

INTRODUCTION

The application of external irradiation to the abdomen is associated with morphological and functional changes in the intestine (Mohiuddin et al, 1978; Timmermans et al, 1977; Yasumoto and Sugiyama, 1980). Abnormalities in the uptake of glucose, leucine, fatty acids and bile acids appear 3 days following 600 rads and persist even at two weeks (Thomson et al, 1983; 1984a,b). However, the uptake of some probes such as cholesterol is unchanged (Thomson et al, 1984a). The mechanism of these changes in transport function following irradiation has not been elucidated. Changes in the fluidity of membranes may be associated with alterations in their lipid composition and function (Schachter, 1984; Stubbs and Smith, 1984). The composition, fluidity and function of some non-intestinal membranes change following irradiation (Fonck et al, 1982a,b; Wolter and Konings, 1982). Accordingly, these studies were undertaken to assess the possible changes in morphology and in the lipid composition of jejunal and ileal brush border membranes at 3, 7, 14 and 28 days following the animals' exposure to 600 rads abdominal irradiation. The results of these studies may explain the previously reported changes in intestinal permeability following irradiation with the same dose and time intervals used in the transport studies.

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[1] A version of this chapter has been published. Keelan, M., Cheeseman, C., Walker, K., and Thomson, A.B.R. 1986. Radiation Research 105:84-96.

METHODS

Studies were performed on female Sprague-Dawley rats weighing 175-225 g. The method used to irradiate the abdomen from a ^{137}Cs source has been described (Thomson et al, 1983). One set of 30 animals was anaesthetized with rhompin/ketamine (2:1, 0.2 ml/300 g body weight) and exposed to 600 rads of abdominal radiation. Four groups of 6-8 animals were sacrificed by ketamine injection (1 ml/300g body weight) at 3, 7/8, 14 or 28 days later. A second set of 30 animals was anaesthetized only and sacrificed on the same days as the irradiated group. These animals served as the control groups. Jejunum and ileum of control and irradiated rats were examined concurrently.

The procedures for obtaining mucosa, isolation of BBM, marker analyses, lipid analyses, and studying villus morphology are described in detail in Appendices 1 - 4.

All data was expressed as mean ± standard error of the mean. A minimum of six animals were present in each group. Each brush border membrane preparation was obtained from one animal. The control groups were tested using one-way analysis of variance, and then pooled together. The unpaired t-test was used for each group (control or irradiated) for each variable with membranes as repetitions. The unpaired t-test is equal to the one-way analysis of variance test for multiple comparisons of the means. The unpaired t-test was chosen because only specific comparisons were of interest, i.e. whether the irradiated group was different from the control group.

RESULTS

1. Marker Analyses

At day 3 following 600 rads, there were reduced values of jejunal mucosal scrapings wet weight, sucrase activity, alkaline phosphatase activity, and alkaline phosphatase/sucrase, but none of these differences were statistically significant when compared with the values obtained in the control rats. There were no significant differences in the jejunum of control and 7/8,14 or 28 day post-irradiation animals in the wet weight of mucosal scrapings, protein content, sucrase or alkaline phosphatase activity, or in the ratio of alkaline phosphatase/sucrase. -glucuronidase activity was similar in control and irradiated animals. No DNA was present in the BBM of control and irradiated animals (Table V-1).

In the ileum of irradiated animals there was a significant increase in the alkaline phosphatase activity and the alkaline phosphatase/sucrase ratio 3, 7/8 and 28 days post-irradiation (Table V-2). Wet weight of mucosal scrapings, protein, sucrase activity and -glucuronidase activity were similar in control and irradiated animals. No DNA was present in the ileal BBM of control and irradiated animals.

2. Lipid Analyses

In the jejunum of irradiated rats, the lipid composition of the BBM was similar to values obtained in control animals when examined 7/8,14 and 28 days after irradiation. The only exception was the decreased total free fatty acid content in the 3 day post-irradiation animals, which contributes to the lower lipid/protein ratio (Table V-3). In the ileum of irradiated animals there was a significant reduction in BBM total free fatty acids at day 3 and 14 after abdominal irradiation.

There were no changes in total bile acids, or cholesterol content. Only in the day 3 post-irradiation animals was the total phospholipid content increased as compared to control animals, which resulted in an increased phospholipid/cholesterol ratio (Tables V-3 and V-4). Days 7/8, 14, and 28 had similar levels of total phospholipid and phospholipid/cholesterol ratios.

When the phospholipids in the jejunal BBM were expressed as nmoles/mg protein (Table V-5), there was a significant reduction in lysolecithin, sphingomyelin, phosphatidic acid, phosphatidyl serine, and phosphatidyl ethanolamine in the irradiated animals, but these changes did not affect the overall choline and amine phospholipid composition. The 14 day post-irradiation animals also contained less phosphatidyl inositol (Table V-5). When the jejunal BBM phospholipids were expressed as a percentage of the total, no significant differences were observed between control and irradiated animals (Table V-5).

Many changes in phospholipid composition are associated with irradiation in the ileal BBM, when expressed as nmoles/mg protein. Lysolecithin was lower at 3, 7/8 and 14 days post-irradiation, but was higher at 28 days post-irradiation. Lecithin was increased 3 and 7/8 days post-irradiation but returned to control levels by 14 days post-irradiation. Sphingomyelin was increased only in 3 day post-irradiation animals. The changes observed in these three choline phospholipids resulted in a quantitative increase in the choline phospholipid content of ileal BBM 3 days after irradiation. Phosphatidyl ethanolamine was increased and phosphatidyl serine was decreased in the irradiated animals, with the exception in the 14 day post-irradiation animals, in which the phosphatidyl serine content was increased. The changes observed in these two amine phospholipids resulted in an overall increase in the ileal BBM amine phospholipid content of the irradiated animals. Phosphatidyl inositol was increased while phosphatidic acid content was decreased in the irradiated animals. None of these changes in phospholipid distribution had any effect on the ratio of choline/amine phospholipids. Very few changes were observed when the phospholipids were expressed as percentage of total phospholipid (Table V-6).

3. Intestinal Morphology

a. Effect of Abdominal Irradiation on Crypts, Villi, and Surface Area

Following irradiation, the jejunal crypt depth was unchanged except at day 14 (Table V-7). Villus height declined at day 3, then rose at day 7/8 and 14 ($p<0.05$). Jejunal villus surface area followed the same pattern of changes observed with villus height. The jejunal mucosal surface area fell approximately 33% 3 and 7/8 days following 600 rads, but then returned to normal. Changes in mucosal surface area were due to changes in villus surface area and villus density (number of villi/mm^2 serosa).

Ileal villus morphology was not affected by abdominal irradiation (Table V-8).

Jejunal microvillus height and the number of microvilli per m were not affected by abdominal irradiation at 600 rads (Table V-7). The height of ileal microvilli was decreased only at 7/8 days post-abdominal irradiation (Table V-8). The number of microvilli per m was significantly increased only at 3 days following 600 rads irradiation.

b. Qualitative Comparison of Morphological Measurements

Different end-points were used to assess intestinal morphology. It was clear that changes in villus height were not necessarily associated with changes in crypt depth, villus surface area, mucosal surface area or microvillus height (Tables V-7 and V-8). Following 600 rads abdominal radiation, the jejunal villus height, villus surface area and mucosal surface area declined on day 3. The reduced jejunal mucosal surface area on day 7/8 was associated with an increased height of the villus and the villus surface area, but reduced villus density (number of villi/mm^2 serosa). At 14 days following 600 rads, the height of the villus and the villus surface area were increased whereas the mucosal surface area was unchanged. Variations in the number of villi/mm^2 serosa explain the different patterns observed in mucosal and villus surface areas.

No consistent relationship was found between changes in the depth of the crypts and the villus height, villus surface area or mucosal surface area. For example, on day 14 following 600 rads, the jejunal crypt depth was below normal, whereas the mucosal surface area was normal. Changes in the height of the microvilli was not necessarily associated with a change in the height of the villus. For example, following 600 rads, the height of the jejunal villi was reduced, but the microvillus height was unchanged.

In general, morphological parameters of the jejunum fell 3 days after abdominal irradiation, whereas in the ileum villus height, villus surface area, and mucosal surface area were unchanged. There were relatively few morphological changes in the ileum at day 7/8 or 14 following 600 rads, whereas increases in villus height and villus surface area were noted in the jejunum.

c. Comparison of Changes in Intestinal Morphology and Brush Border Membrane Marker Enzymes and Lipid Analysis

Detailed analyses of brush border membrane enzyme markers and lipid composition were performed following 600 rads abdominal irradiaton. Jejunal brush border membrane sucrase and alkaline phosphatase activities fell (though not significantly below control values) on day 3 following 600 rads, and at this time the villus height, villus surface area, and mucosal surface area had declined (Tables V-1 and V-7). On days 7/8 and 14, the jejunal brush border membrane sucrase and alkaline phosphatase activities had returned to normal while the villus height and villus surface area were increased above control values at these times. In the ileum, the brush border membrane sucrase activity was reduced on day 3 following abdominal irradiation, yet the alkaline phosphatase activity was increased (Table V-2). Despite these changes in enzyme markers in the brush border membrane, the ileal villus height, villus surface area, and mucosal surface area were unchanged (Tables V-7 and V-8). Linear regression analysis demonstrated a significant correlation between villus height and villus surface area ($y=27.9 + 0.95x$, $r=0.989$, $p<0.01$), villus height and sucrase activity ($y=-32.7 + 2.99x$, $r=0.966$, $p<0.01$), and between villus height and alkaline phosphatase activity ($y=-547 + 2.89x$, $r= 0.981$, $p<0.01$). Changes in morphology were related to changes in BBM enzyme activity.

Quantitative expression of the lipid composition (nmoles/mg protein) in the jejunal brush border membrane was unaffected by irradiation except for the decreased free fatty acid content at day 3. In the ileal brush border membrane, there were quantitative decreases in free fatty acid content and increases in phospholipid content at day 3. The quantitative lipid changes observed in the jejunum and ileum returned to control levels at day 7/8 and 14 post-irradiation. Many morphological changes were observed throughout the post-irradiation period but could not be correlated to the alterations in membrane lipid content.

Further examination of the individual phospholipids revealed no alterations in the qualitative (percent) proportions of phospholipids in the jejunal brush border membrane (not shown). This was also true for the ileum, with the exception of a qualitative increase in lecithin at day 7/8 which returned to control levels by day 14 post irradiation. Several minor quantitative (nmoles/mg protein) phospholipid changes occurred following irradiation with 600 rads in the jejunum (Table V-7). The overall jejunal choline and amine phospholipid composition was not altered following irradiation. There were many more alterations observed in the ileal brush border membrane phospholipid composition. Both lecithin and sphingomyelin (choline phospholipids) as well as phosphatidyl ethanolamine (amine phospholipid) were present in larger amounts by day 3 following irradiation, but returned to control levels by day 7/8 or 14. The early increases resulted in significant increases in both total choline and total amine phospholipids such that the choline/amine phospholipid ratio did not change. Minor changes were also observed with the other ileal phospholipids (Table V-6). Alterations in phospholipid composition were not correlated with the morphological changes observed.

DISCUSSION

Abdominal irradiation was associated with significant changes in jejunal morphology (Table V-7). Thus, following abdominal irradiation, the changes in morphology were associated with significant changes in the brush border membrane phospholipid composition but are not generally associated with significant changes in the brush border membrane enzyme markers, or in the brush border total lipids. It was at day 3, 7/8 and 28 following 600 rads that the ileal brush border membrane alkaline phosphatase activity was increased (Table V-2) and amine phospholipids were increased (Table V-6), but at this time the villus height, villus surface area and mucosal surface area were normal (Tables V-7 and V-8). The changes in the jejunal morphology following 600 rads were not associated with consistent changes in membrane total lipids. Thus changes in intestinal morphology did not consistently correlate with changes in the marker enzyme activity or the total lipid content of the BBM, but were associated with changes in phospholipid composition.

The changes in carrier-mediated and passive transport following abdominal irradiation were not consistently associated with changes in morphology. For example, hexose uptake was reduced 3 days following 600 rads (Thomson et al, 1983) when the height of the villi, villus surface area and mucosal surface area was reduced (Table V-7). However, hexose absorption remained variably abnormal despite the increased villus height and villus surface area at 7/8 days following 600 rads. Changes in passive permeation were not consistently associated with changes in morphology. For example, 14 days following 600 rads, the uptake of fatty acids was decreased (Thomson et al, 1984a), despite the normal mucosal surface area. It is unknown what proportion of the directionally variable changes in lipid uptake following different doses of irradiation occur due to changes in certain portions of the villi, without overall alterations in the morphological parameters. It is possible that changes in the overall villus height and villus surface area or mucosal surface area will not be correlated with changes in uptake, since the proportion of the villus used for uptake of a given probe may change, thereby altering the in vitro uptake of the probe in question. Furthermore, the lipid analysis of the brush border membrane was obtained from the isolation of the entire villus membrane. Although there were no changes in the lipid composition of selected portions of the villus, it is unknown whether there were changes in the lipid composition of that portion of the membrane through which lipid permeation proceeded.

Other studies have suggested that composition of membranes may change following irradiation (Fonck et al, 1982a,b; Wolter and Konings, 1982). The change in the lipid composition of the membranes may alter membrane fluidity (Schachter, 1984; Stubbs and Smith, 1984). Indeed, intestinal permeability is altered under a number of experimental circumstances such as aging (Bowman and Rosenberg, 1983; Hollander and Morgan, 1979a,b; Holt and Dominguez, 1981; Jakob and Penzes, 1981; Klimas, 1968; Pelz et al, 1968; Thomson, 1979, 1981a) and with streptozotocin-induced diabetes mellitus (Thomson et al, 1983; Olsen and Rogers, 1970; Thomson, 1980; 1981b). The BBM composition of lipids also changes with aging (Keelan et al, 1985) with diabetes mellitus (Keelan et al, 1984). Thus, following abdominal irradiation, it remains unclear what the mechanism is for changes in the passive permeability properties of the intestine, since the changes in lipid uptake do not appear to be clearly associated with changes in intestinal morphology, or BBM enzyme or total lipid composition. However, the variations in phospholipid composition observed following irradiation may be associated with altered permeability of the jejunal and ileal brush border membrane. In addition, the quantitative increase in ileal membrane phospholipid increases the phospholipid/cholesterol ratio at 3 days post-irradiation and may be a possible mechanism by which the permeability of the ileal brush border membrane is altered.

Plasma membranes are thought to be a sensitive target site where radiation-induced injury first presents itself (Chandra and Stefani, 1981). It is well established that radiation induces lipid peroxidation through the production of free radicals which attack the fatty acid chain of phospholipids (Petkau and Chelak, 1976). A decrease in unsaturated fatty acid composition is associated with irradiated lipids with a corresponding increase in peroxidation products (De and Aiyar, 1978; Hammer and Wills, 1979). Future studies will determine the effect of external irradiation on the brush border membrane fatty acid chains of the phospholipids. Studies by Khar and co-workers (Khar et al, 1982) have shown that the lipid composition of yeast membranes can be altered such that the membrane becomes resistant to the transport changes that are observed following irradiation. Further studies will be conducted in an attempt to alter the brush border membrane lipid composition with diet in order to decrease the radiation sensitivity of the gut.

TABLE V-1 EFFECT OF IRRADIATION ON ENZYME MARKER ANALYSIS OF JEJUNAL BRUSH BORDER MEMBRANES

Membrane Marker	Control	Post Abdominal Irradiation (600 RADS)			
		Day 3	Day 7/8	Day 14	Day 28
Mucosal scrapings, g wet weight, /40 cm length	1.17±0.03	0.88±0.07	1.43±0.11	1.38±0.06	1.31±0.08
Protein, mg/g wet weight	2.32±0.1	2.38±0.19	2.18±0.10	2.15±0.17	2.34±0.11
Sucrase, U/g protein	1232±54	707±75	1330±80	1134±59	1122±137
Alkaline phosphatase, U/g protein	757±53	528±88	1056±156	984±174	1021±113
Alkaline phosphatase/ sucrase	0.635±0.047	0.873±0.140	0.829±0.117	0.852±0.138	0.707±0.079
β-glucuronidase, U/g protein	0.186±0.012	0.153±0.014	0.157±0.008	0.243±0.043	0.222±0.032
DNA, µg/mg protein	0	0	0	0	0

No significant difference (p>0.05), irradiated versus control animals.

TABLE V-2 EFFECT OF IRRADIATION ON ENZYME MARKER ANALYSIS OF ILEAL BRUSH BORDER MEMBRANES

Membrane Marker	Control	Post Abdominal Irradiation (600 RADS)			
		Day 3	Day 7/8	Day 14	Day 28
Mucosal scrapings, g wet weight /40 cm length	0.71±0.02	0.57±0.06	0.85±0.08	0.90±0.05	0.83±0.04
Protein, mg/g wet weight	2.57±0.09	2.22±0.24	1.72±0.16	2.06±0.11	2.44±0.17
Sucrase, U/g protein	374±20	188±38	308±52	282±39	352±44
Alkaline phosphatase, U/g protein	26±2	60±10*	62±21*	30±7	75±24*
Alkaline phosphatase/ sucrase	0.075±0.006	0.440±0.080*	0.262±0.086*	0.154±0.061	0.288±0.063*
β-glucuronidase, U/g protein	0.369±0.031	0.275±0.038	0.360±0.024	0.348±0.037	0.404±0.051
DNA, µg/mg protein	0	0	0	0	0

* $p < 0.05$, Irradiated versus control animals.

TABLE V-3 EFFECT OF IRRADIATION ON LIPID ANALYSIS OF JEJUNAL BRUSH BORDER MEMBRANES

Lipid, nmol/mg protein	Control	Post Abdominal Irradiation (600 RADS)			
		Day 3	Day 7/8	Day 14	Day 28
Total lipid	979 ± 45	710 ± 24*	915 ± 60	820 ± 80	1010 ± 69
Total free fatty acids	262 ± 17	136 ± 26*	287 ± 37	216 ± 26	282 ± 39
Total bile acids	0.5 ± 0.2	0.2 ± 0.2	0.4 ± 0.2	0.7 ± 0.4	0.8 ± 0.4
Total phospholipids	397 ± 32	329 ± 20	384 ± 36	315 ± 98	406 ± 56
Cholesterol					
total	302 ± 18	245 ± 10	269 ± 17	248 ± 18	281 ± 15
free	282 ± 7	220 ± 9	257 ± 13	229 ± 18	263 ± 6
esters	21 ± 4	26 ± 8	12 ± 5	20 ± 6	18 ± 11
Total phospholipid/ total cholesterol	1.3 ± 0.1^{+}	1.3 ± 0.1	1.1 ± 0.2	1.3 ± 0.5	1.3 ± 0.2

* $p<0.05$, irradiated versus control animals.

TABLE V-4 EFFECT OF IRRADIATION ON LIPID ANALYSIS OF ILEAL BRUSH BORDER MEMBRANES

Lipid nmol/mg protein	Control	Post Abdominal Irradiation (600 RADS)			
		Day 3	Day 7/8	Day 14	Day 28
Total lipid	877 ± 21	870 ± 48	928 ± 51	750 ± 52*	1021 ± 108
Total free fatty acids	279 ± 18	85 ± 31*	274 ± 29	123 ± 31*	252 ± 41
Total bile acids	0.2 ± 0.1	0	0.3 ± 0.2	0.5 ± 0.3	0.5 ± 0.4
Total phospholipids	256 ± 19	498 ± 38*	343 ± 20	341 ± 14	317 ± 32
Cholesterol,					
total	336 ± 11	271 ± 29	321 ± 32	290 ± 21	325 ± 19
free	309 ± 10	248 ± 21	295 ± 22	264 ± 17	301 ± 9
esters	27 ± 5	23 ± 14	26 ± 16	26 ± 9	24 ± 13
Total phospholipid/ total cholesterol	0.8 ± 0.1	1.6 ± 0.1*	1.1 ± 0.1	1.2 ± 0.1	0.9 ± 0.1

* $p < 0.05$, irradiated versus control animals.

TABLE V-5 EFFECT OF IRRADIATION ON PHOSPHOLIPID COMPOSITION OF JEJUNAL BRUSH BORDER MEMBRANES

	Control	Post Abdominal Irradiation (600 RADS)			
Phospholipid		Day 3	Day 7/8	Day 14	Day 28
Lecithin,					
nmoles/mg protein	187.4±15.1	170.9±10.5	168.2±15.7	170.6±48.0	189.3±26.2
%	48.6±1.8	52.0±1.7	43.8±1.7	47.0±1.1	46.6±1.6
Lysolecithin,					
nmoles/mg protein	0.4±0.03	ND*	4.2±0.4*	1.3±0.4*	2.4±0.3*
%	0.1±0.1	ND	1.1±1.1	0.4±0.4	0.6±0.6
Sphingomyelin,					
nmoles/mg protein	49.3±4.0	43.1±2.6	55.3±5.2	51.3±16.0	46.3±6.4
%	12.8±1.0	13.1±2.0	14.4±2.4	16.3±2.0	11.4±1.6
Phosphatidyl serine,					
nmoles/mg protein	4.2±0.3	ND*	15.7±1.5*	ND*	ND*
%	1.1±0.5	ND	4.1±2.0	ND	ND
Phosphatidyl ethanolamine,					
nmoles/mg protein	122.2±9.8	92.3±5.7	113.7±10.6	107.1±33.3	138.1±19.1
%	31.7±1.6	28.1±1.9	29.6±2.6	33.3±2.0	34.0±1.1
Phosphatidyl inositol,					
nmoles/mg protein	20.0±1.6	22.7±1.4	20.0±1.9	9.8±3.0*	17.1±2.4
%	5.2±0.8	6.9±1.1	5.2±1.0	3.1±1.1	4.2±1.3
Phosphatidic acid,					
nmoles/mg protein	3.5±0.3	ND*	6.9±0.6*	ND*	ND*
%	0.9±0.4	ND	1.8±1.1	ND	ND
Choline Phospholipid,					
nmoles/mg protein	233.0±19.8	213.7±13.1	227.7±21.3	200.2±62.4	238.8±33.1
%	61.3±1.6	65.0±2.2	59.3±2.7	63.6±2.2	58.8±1.8
Amine Phospholipid,					
nmoles/mg protein	126.4±10.2	92.3±5.7	134.0±12.5	104.9±32.7	152.7±21.2
%	32.8±1.6	28.1±1.9	34.9±3.3	33.3±2.0	37.6±2.2
Choline/Amine Phospholipid	2.1±0.1	2.2±0.2	2.0±0.3	2.1±0.3	1.6 ± 0.1

ND, none detected

* $p < 0.05$, irradiated versus control animals

TABLE V-6 EFFECT OF IRRADIATION ON PHOSPHOLIPID COMPOSITION OF ILEAL BRUSH BORDER MEMBRANES

Phospholipid	Control	Post Abdominal Irradiation (600 RADS) Day 3	Day 7/8	Day 14	Day 28
Lecithin,					
nmoles/mg protein	105.2±7.8	233.6±17.7*	161.5±9.4*	133.5±5.4	128.3±12.9
%	41.1±1.1	46.9±3.1	47.1±2.3*	39.2±2.7	40.4±2.3
Lysolecithin,					
nmoles/mg protein	2.3±0.2	ND*	ND*	ND*	3.5±0.3*
%	0.9±0.5	ND	ND	ND	1.1±1.0
Sphingomyelin,					
nmoles/mg protein	61.7±4.6	94.6±7.2*	60.4±2.5	61.7±2.5	63.8±6.4
%	24.1±1.5	19.0±3.3	17.6±2.2	18.1±3.3	20.1±2.8
Phosphatidyl serine,					
nmoles/mg protein	3.3±0.3	ND*	1.7±0.1*	6.1±0.2*	ND*
%	1.3±0.5	ND	0.5±0.5	1.8±1.7	ND
Phosphatidyl ethanolamine,					
nmoles/mg protein	74.7±5.6	144.9±11.0*	108.7±6.3*	126.7±5.1*	109.8±11.0*
%	29.2±1.5	29.1±4.2	31.7±2.2	37.2±4.0	34.6±2.2
Phosphatidyl inositol,					
nmoles/mg protein	7.4±0.6	24.9±1.9*	8.9±0.5	29.6±1.2*	12.4±1.2*
%	2.9±0.5	5.0±1.4	2.6±1.3	8.7±1.7	3.9±1.4
Phosphatidic acid,					
nmoles/mg protein	1.5±0.1	ND*	ND*	ND*	ND*
%	0.6±0.4	ND	ND	ND	ND
Choline Phospholipid,					
nmoles/mg protein	168.6±12.6	299.8±50.4*	223.7±22.4	195.2±13.6	195.3±39.1
%	65.9±1.8	60.2±2.9	65.2±2.3	57.3±4.7	61.5±3.4
Amine Phospholipid,					
nmoles/mg protein	77.8±5.8	152.4±25.9*	110.5±11.0*	132.9±9.2*	109.8±22.0*
%	30.4±1.5	30.6±4.4	32.2±2.1	39.0±4.4	34.6±2.2
Choline/Amine Phospholipid	2.7±0.3	2.0±0.2	2.1±0.2	2.0±0.5	1.9±0.2

ND none detected

* $p < 0.05$, irradiated versus control animals

TABLE V-7 EFFECT OF IRRADIATION ON JEJUNAL MORPHOLOGY

Morphological Parameter	Control	Post-Abdominal Irradiation (600 Rads)		
		Day 3	DAY 7/8	DAY 14
Crypt depth, μm	108 ± 6	112 ± 6	108 ± 10	71 ± 3*
Villus:				
height, μm	433 ± 18	320 ± 15*	579 ± 21*	523 ± 27*
width at $^1/_2$height, μM	112 ± 5	106 ± 8	107 ± 4	126 ± 5
bottom width, μm	98 ± 6	110 ± 6*	194 ± 25*	109 ± 7
surface area, μm^2/villus	446 ± 19	331 ± 20*	543 ± 20*	546 ± 28*
No. of cells/villus	131 ± 3	74 ± 3*	166 ± 8*	181 ± 8*
No. of villi/mm serosal length	10.7 ± 0.5	9.4 ± 0.5	5.7 ± 0.7*	9.7 ± 1.0
No. of villi/mm^2 serosa	28.39 ± 1.41	26.78 ± 1.29	16.19 ± 1.96*	27.80 ± 2.85
Mucosal surface area, mm^2/mm^2 serosa	13.38 ± 0.93	8.93 ± 0.76*	8.68 ± 0.91*	15.26 ± 1.79
Microvillus height, μm	1.28 ± 0.12	1.61 ± 0.10	1.21 ± 0.05	1.31 ± 0.09
No. of microvilli/μm	6.1 ± 0.2	5.6 ± 0.3	6.4 ± 0.3	5.3 ± 0.3

* $p < 0.05$, irradiated versus control animals

TABLE V- 8 EFFECT OF IRRADIATION ON ILEAL MORPHOLOGY

Morphological Parameter	Control	Post-Abdominal Irradiation (600 Rads)		
		Day 3	Day 7/8	Day 14
Crypt depth, μm	103 ± 4	136 ± 6	71 ± 5	74 ± 4
Villus:				
height, μm	222 ± 6	183 ± 5	250 ± 14	215 ± 10
width at $^1/_2$height, μm	97 ± 2	87 ± 2	122 ± 5	91 ± 4
bottom width, μm	94 ± 3	91 ± 2	124 ± 7^{*+}	78 ± 4
surface area, μm^2/villus	218 ± 7	193 ± 9	285 ± 16	232 ± 17
No. of cells/villus	63 ± 2^{+}	35 ± 2*	92 ± 16*	84 ± 3*
No. of villi/mm serosal length	10.9 ± 0.3	11.1 ± 0.4	8.4 ± 0.5	13.7 ± 1.1
No. of villi/mm^2 serosa	34.67 ± 1.03	37.07 ± 1.02	22.60 ± 1.45*	38.34 ± 2.93
Mucosal surface area, mm^2/mm^2 serosa	7.47 ± 0.27	5.99 ± 0.31	6.81 ± 0.64	8.40 ± 0.49
Microvillus height, μm	1.10 ± 0.05	1.23 ± 0.12	0.69 ± 0.06*	1.11 ± 0.08
No. of microvilli/μm	5.9 ± 0.2	7.1 ± 0.4*	6.8 ± 0.4	5.9 ± 0.2

* $p < 0.05$, irradiated versus control animals

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CHAPTER VI

SUMMARIZING DISCUSSION

Intestinal adaptation does indeed occur via changes in the activity of brush border membrane (BBM) enzymes, BBM lipid composition and villus morphology. However, what is the interrelationship between the digestive, absorptive, structural and biochemical alterations?

The pattern of adaptation for the BBM enzymes was different in each of the animal models studied. Jejunal and ileal BBM sucrase (S) activity progressively increased with aging from weanling-young-mature animals. BBM alkaline phosphatase (AP) activity at both sites was highest in young animals and similar in weanling and mature animals. Diabetes was not associated with any change in S or AP. Fasting did not affect jejunal BBM enzyme activity in control animals, but was associated with an increase in S in diabetic animals. Chronic ethanol consumption increased S activity and reduced AP activity in both the jejunal and ileal BBM. External abdominal irradiation did not alter jejunal BBM enzyme activities, but did increase AP activity in the ileum. Enzyme activity was expressed in each of the studies as specific activity (U/g protein), not total activity (U/total BBM). Specific activity was thought to be a more appropriate measure of enzyme activity since the total amount of BBM isolated varied between animals and between intestinal sites, yet protein content was constant when expressed per gram wet weight. Changes in the total activity of the enzymes reflect a quantitative difference dependent on the amount of BBM present. Specific activity is related to the amount of protein present and is not affected by fluctuations in the quantitative amount of BBM isolated. The BBM purification and yields were similar in the different test groups. These studies did not distinguish whether the alterations in enzyme activity were due to: 1) changes in the proportion of enzyme protein relative to the total protein, or 2) whether the proportion of enzyme protein relative to total protein was constant such that changes in enzyme activity reflect an alteration in the functional characteristics and properties of the enzyme, and not just a change in the amount of protein present.

Autoradiography studies have revealed that only the upper 1/3 of the villus is capable of nutrient absorption (Smith, 1981; Haglund et al, 1973). Cells become biochemically and functionally mature as they migrate up the villus (Chapter I) resulting in a mixed cell population. If the biochemical maturity of the cells along the villus is altered (as assessed by measuring AP and S of the entire villus mixed cell population), it is possible that the population of functionally mature cells is also altered, thereby providing one possible mechanism by which nutrient transport could be altered in the animal models studied. The ratio of AP/S is thought to be a marker of cell maturity (Hartman et al, 1982) with a reduced ratio of AP/S suggestive of a greater proportion of mature cells. A more mature cell population would be expected to be associated with an increased ability to transport nutrients. AP/S was reduced with aging and chronic ethanol consumption which suggests a more mature cell population along the villus. Although active nutrient transport was increased with aging, passive nutrient transport was reduced (Table VI-1). In chronic ethanol consumption, both active glucose and passive lipid nutrient transport were reduced.

Changes in cell maturity do not adequately explain the changes in nutrient transport observed in these two models. An immature cell population would be expected to be associated with a reduced ability to transport nutrients. A higher AP/S ratio was observed in the ileal BBM following irradiation, which suggests a predominantly more immature cell population along the villus. Both active and passive nutrient transport were reduced following external abdominal irradiation (Table VI-1). In this model, cell immaturity correlates well with the reduced transport function observed. The maturity of the villus cell population was unaffected by diabetes as reflected by the unchanged ratio of AP/S as compared with non-diabetic control rats. Both active and passive nutrient transport were increased in the diabetic animal (Table VI-1). Since cell maturity (as assessed by AP/S) was constant, it does not explain the increase in nutrient transport previously reported. It is clear that changes in cell maturity as assessed by the AP/S ratio may provide a possible mechanism for alterations in nutrient transport following irradiation, but does not explain the functional changes observed in the other animal models tested.

Intestinal adaptation involved alterations in the BBM lipid composition in each of the models studied (Table VI-1). Increases in the free fatty acid or phospholipid content of the BBM would be expected to be associated with a more permeable membrane, which would be reflected in the increased passive transport of nutrients. Alterations in lipid composition may also influence active transport by altering the environment around the carrier protein. Aging was associated with an increased amount of free fatty acid and cholesterol ester in jejunal BBM, while total phospholipid and the ratio of phospholipid/cholesterol

(PL/C) were increased in the ileal BBM. Diabetes was also associated with a higher amount of total phospholipid and a higher PL/C. Fasting produced a similar increase in total phospholipid and PL/C in control jejunal BBM, but the cholesterol content was only slightly increased and the free fatty acid content was decreased in the jejunal BBM of fasting diabetic animals. Chronic ethanol consumption reduced the jejunal BBM phospholipid content and PL/C. The free fatty acid content of jejunal and ileal BBM was reduced 3 days post-irradiation. Only the ileal BBM contained a higher amount of phospholipid and a higher PL/C up to 14 days post-irradiation. A higher PL/C was associated with an increase in passive transport in diabetes, but not with aging or following irradiation (Table VI-1). A reduced PL/C was observed in animals chronically fed ethanol which correlated well with a reduction in passive transport. An increased PL/C was associated with increased active transport in diabetes and with aging, but not following irradiation. A reduced PL/C was associated with a reduction in active transport in ethanol-fed animals. Since the amount of membrane cholesterol appears to be fairly constant, it appears to be the phospholipid content that has some relationship with the transport changes observed in the animal models.

In every model tested, phospholipid content was altered in the process of adaptation. BBM cholesterol content remained remarkably constant, which has led other workers to the suggestion that the cholesterol content of the BBM must be regulated (Brasitus & Schachter, 1982). I hypothesize that since it is the phospholipids that are changing, it is the phospholipid content and composition that are regulated in an adaptive response to the animal model manipulation.

Since the phospholipid content was altered in each of the models studied, it was of interest to determine which phospholipid class was involved. The type of phospholipid present influences the packing and structure of the BBM. Sphingomyelin is a fairly rigid molecule and its presence confers an increase in rigidity of the membrane. Lecithin has a large polar head group which allows for greater mobility of its fatty acid chains and is thereby associated with a greater membrane fluidity. Phosphatidyl ethanolamine has a small polar head group, therefore the movement of its fatty acid chains is more restricted and tightly packed as compared to those of lecithin. In the young animals, the choline phospholipids (lecithin, sphingomyelin) were increased, while the amine phospholipids (primarily phosphatidyl ethanolamine) were similar to those in weanling animals. The choline/amine phospholipid ratio was higher in the jejunal BBM of young animals, but was not affected by age in the ileal BBM. When compared with young rabbits, mature animals have a lower choline and a higher amine phospholipid content, such that the choline/amine phospholipid ratio is similar to that in weanling animals. Diabetes was associated with an increase in both choline (lecithin) and amine (phosphatidyl ethanolamine) phospholipids. Fasting was also associated with an increase in lecithin and phosphatidyl ethanolamine in control animals. In chronic ethanol consumption, jejunal BBM choline (lecithin) was reduced significantly such that the choline/amine phospholipid ratio was also reduced. Minor changes in phospholipid composition were observed in jejunal BBM following irradiation. In the ileal BBM choline (lecithin, sphingomyelin) and amine (phosphatidyl ethanolamine) were both increased at 3 days post-irradiation, but only the amine phospholipids remain elevated even at day 28 post-irradiation. The effects of altering the phospholipid composition on membrane function are not well understood. No simple relationship can be described between any specific phospholipid and transport function.

Variations in membrane cholesterol content, phospholipid composition and fatty acid composition can be modified in mammalian cells (Spector and Yorek, 1985). Changes in membrane lipid composition may alter the physical properties of the membrane and thereby influence many cellular functions including the activity of membrane-bound enzymes, carrier-mediated transport, passive membrane transport, and many others.

Changes in phospholipid content and composition may influence the activity of intrinsic BBM enzymes such as alkaline phosphatase (AP). Seetharum and co-workers (1985) demonstrated that AP was bound to the membrane primarily by hydrophobic interactions with the membrane phospholipids without the absolute requirement of the polar head group. The activity of AP was, however, influenced by the secondary interaction with choline head groups. When the polar head group was removed with phospholipase D, the activity of AP decreased. Upon the addition of free choline, AP activity was restored. In animal models where choline content was reduced (mature versus young rabbits, ethanol-fed rabbits), AP activity was also decreased. The inverse was not always true since an increase in the choline phospholipids (young versus weanling rabbits, diabetic rats, fasting control rats, irradiated rats) was not always associated with an increase in AP activity. Linear regression analysis revealed that BBM enzyme activity was not significantly correlated with changes in phospholipid content or PL/C.

An increase in phospholipid content is thought to be associated with a more fluid membrane, which in turn may be more permeable to passively absorbed nutrients. In the animals where phospholipid content was increased (ileal BBM of mature rabbits and irradiated rats, jejunal BBM of diabetic rats and fasted control rats), the passive absorption of nutrients was not always increased and may in fact be significantly decreased, such as following irradiation. Carrier-mediated nutrient transport may also be influenced by the lipid composition surrounding the carrier by altering its conformation and activity. It is clear that alterations in phospholipid content and composition were associated with changes in both carrier-mediated and passive nutrient transport, although no simple relationship was demonstrated. It is likely that a complex interaction of the many diverse phospholipids affect the properties of the membrane bilayer, which in turn influence the nutrient transporting ability of the BBM.

Free fatty acids are present in the BBM, but their presence is not thought to be due to phospholipid breakdown during membrane purification. There may in fact be a pool of free fatty acids in the BBM which is generated as a result of the continuous turnover of membrane phospholipid fatty acid groups (Spector et al, 1981; Spector and Steinberg, 1967; Pasternak and Bergeron, 1970; Gallaher et al, 1973; D'Souza et al, 1983; Lapetina et al, 1980). Membrane free fatty acids could be direct mediators of functional effects (Spector and Yorek, 1985). The high free fatty acid content could also be due in part to the presence of free fatty acids in transit through the BBM. Free fatty acids may be important for the acylation of some membrane proteins. It has been suggested that the fatty acyl groups may facilitate attachment to the lipid bilayer, channel proteins for recycling or induce conformational changes in the protein (Magee and Schlessinger, 1982).

Fatty acids of the intestinal BBM phospholipids have not been well studied in the models of aging, diabetes, chronic ethanol consumption or irradiation. As discussed in the Introduction (Chapter I), the nature of the phospholipid fatty acid groups greatly influences the physical properties of the membrane and should be studied in the future. It has been suggested that the degree of fatty acid unsaturation may influence the phospholipid head group composition and vice versa (Stubbs and Smith, 1984). Since the alterations in phospholipid head group composition have been observed in each of the animal models studied, it would be of interest to examine the fatty acid composition of the individual phospholipids. Preliminary studies of BBM from control rats reveal that five fatty acids make up 70-85% of the total fatty acids of phosphatidyl choline and phosphatidyl ethanolamine: 16:0, 18:0, 18:1 9, 18:2 6, 20:4 6. The numerous remaining fatty acids are present in less than 2% each. Studies are currently in progress which will examine the fatty acid composition of BBM phospholipids from diabetic rats. Alterations in phospholipid fatty acid composition may greatly influence the passive permeability properties of the BBM as well as the properties of the active transport carrier proteins. Analyses of the BBM phospholipid fatty acids may further aid in the identification of the mechanism by which transport function is altered.

The measurement of mucosal surface area included several aspects of villus architecture not previously considered by most other workers (Appendix 4). Changes in villus height were not always associated with changes in villus surface area or mucosal surface area. Although ileal villus height and villus surface area increase with age from weanling to young rabbits, mucosal surface area is unchanged due to the reduced number of villi per mm^2 serosa (Chapter II). The same pattern was observed for the jejunal morphology of ethanol-fed versus control-ad libitum rabbits (Chapter IV). When compared to weanling rabbits, the jejunal villus height and mucosal surface area of mature rats were similar yet the villus surface area was signficantly increased due to the increase in villus thickness (Chapter II). In the ileum of ethanol-fed rabbits, both villus surface area and mucosal surface area were increased when compared to control-ad libitum rabbits without an increase in villus height due to the increase in villus thickness (Chapter IV). It is evident that other aspects of mucosal architecture, other than villus height, greatly influence both villus and mucosal surface area: villus thickness and villus density (number of villi per mm^2 serosa). Linear regression analysis revealed villus height measurements were significantly correlated to villus surface area (Figure VI-1) and mucosal surface area (Figure VI-2). Villus surface area also correlated well with mucosal surface area (Figure VI-3). Mucosal morphology was not correlated with BBM enzyme activity and lipid composition. Microvillus morphology was not included in the calculation of mucosal surface area. Microvilli increase the surface area of the mucosa approximately 25 times. Jejunal microvillus morphology was not significantly altered with diabetes or following irradiation. Ileal microvillus morphology was altered following irradiation, although the changes here did not reflect changes in mucosal surface area. Thus the lack of changes in villus or mucosal surface area in these animals was not counter-influenced by changes in microvillus height or density.

Jejunal mucosal surface area (MSA) was highest in young animals and similar between weanling and mature animals, while ileal MSA was unaffected by aging. Diabetes was not associated with a change in jejunal MSA when the animals were fed, but MSA was much higher in diabetic than control animals when the animals were fasted. Fasting was associated with a lower MSA only in control animals. Chronic ethanol consumption did not change jejunal MSA but was associated with a higher ileal MSA. Following irradiation, jejunal MSA was reduced up to 7 days later while ileal MSA was unaffected.

The changes in MSA did not correlate well with changes in nutrient transport function. However, the technique used for the assessment of MSA gave only a static measurement of mucosal morphology. In order to more accurately study the relationship between morphology and transport function it would be necessary to measure dynamic morphology, which would allow an assessment of the functional surface area of the villus. This would include only the functional absorptive cells of the upper third of the villus. Dynamic morphology studies would include studies of cell maturity and cell kinetics.

There are two basic mechanisms of absorption:

1) carrier-mediated transport which can be subdivided into active transport (requires energy) and facilitated transport (does not require energy).

2) passive transport which includes transport through tight junctions, water-filled pores and transport through the lipid membrane. The kinetics of these absorption processes may be changed via alterations in the energetics or by blocking the transport process.

Alterations in carrier-mediated transport involves changes in the:

1) Michaelis constant

a) apparent K_m (K_m^*) - includes the effects of unstirred water layers

b) true K_m - could be altered by changes in the structural conformation of the carrier.

2) maximal transport rate (V_{max}) - predominant mechanism by which carrier-mediated transport is altered (Karasov and Diamond, 1983).

The proportion of the alteration by K_m and V_{max} has been described (Dietschy, 1970). The mechanisms of transport change in the animal models studied are shown in Table VI-2. A change in transport at low concentration of solute reflects a change in the K_m, while a change in transport at high concentrations of solute reflects a change in V_{max} and/or the permeability coefficient (P_d).

How does V_{max} change? The presence of more carrier which could be achieved by:

a) increasing the mucosal surface area, and thereby increasing the total number of enterocytes,

b) an increase in the number of transporting enterocytes, without any change in the total number of enterocytes.

c) an increase in the amount of carrier in each cell.

d) alterations in the Na^+ gradient

e) membrane potential

D-glucose and some amino acids (i.e. L-valine, L-alanine, phenylalanine, methionine) can be transported across the BBM via a sodium-dependent mechanism. Alterations in the Na+ gradient would influence the transport of these nutrients. This could be achieved by alterations in the intracellular Na^+ concentration, or an increase in Na^+K^+ATPase activity (Murer and Hildmann, 1984).

How does P_d change?

a) the size of the membrane pores

b) the number of pores in the membrane

c) alterations in the unstirred water layer

d) alterations in the incremental change in free energy (Fw L).

In the animal models studied, previous work has demonstrated that although changes in the characteristics of the unstirred water layer do occur, these changes are insufficient to explain the direction and magnitude of the observed transport changes. Changes in mucosal surface area do not consistently agree with changes in transport. Alterations in the incremental change in free energy are related to the physical properties of the membrane which are mediated by the lipid composition. In each of the models studied, alterations in the lipid composition, in particular the phospholipid composition were observed. These phospholipid changes are likely major contributors to the functional adaptation occurring in each model.

Available evidence which links the changes in membrane lipid composition with altered membrane function is based solely on correlations. This is due to the difficulty in the past of obtaining direct and conclusive evidence for a cause and effect relationship. It has not yet been possible to definitively classify the effects of altering lipid composition to alterations in membrane function according to a consistent pattern. Adaptation is likely a complex process involving many aspects of membrane composition and structure, which result in changes in membrane function.

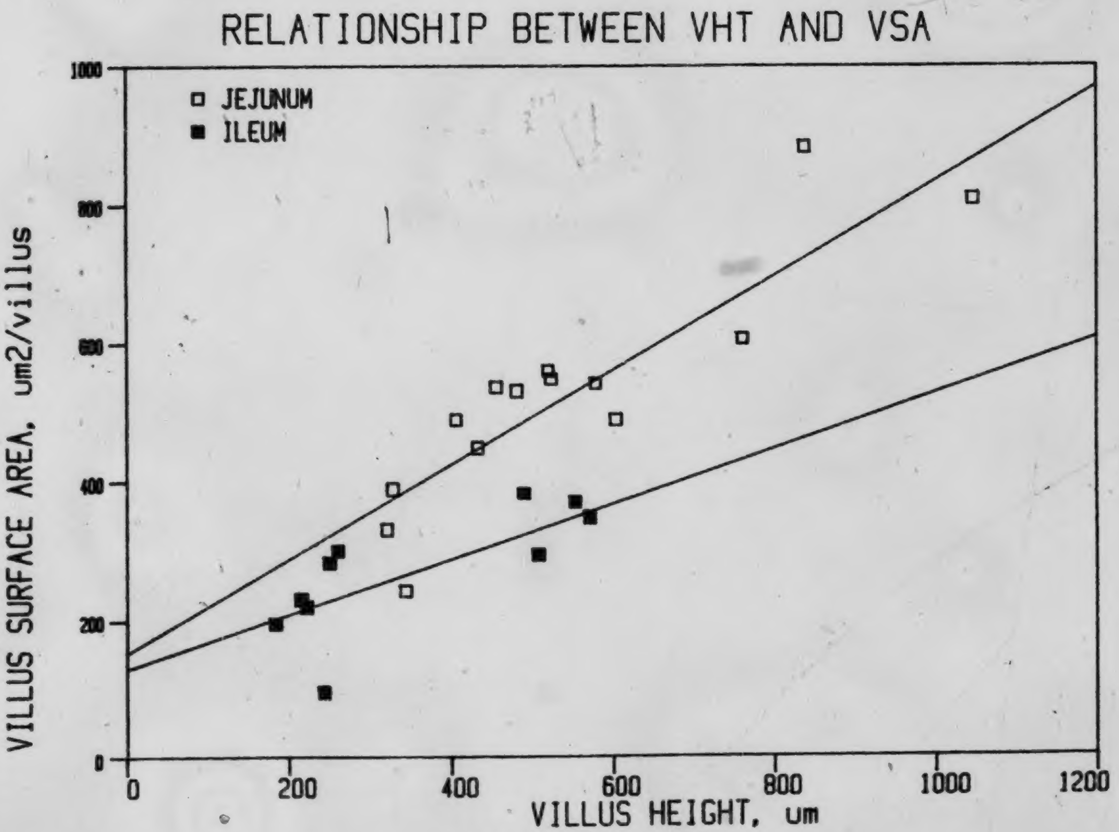

Figure VI-1. Relationship between villus height and villus surface area.

(jejunum: r=0.882, p<0.01; ileum: r=0.744, p<0.01)

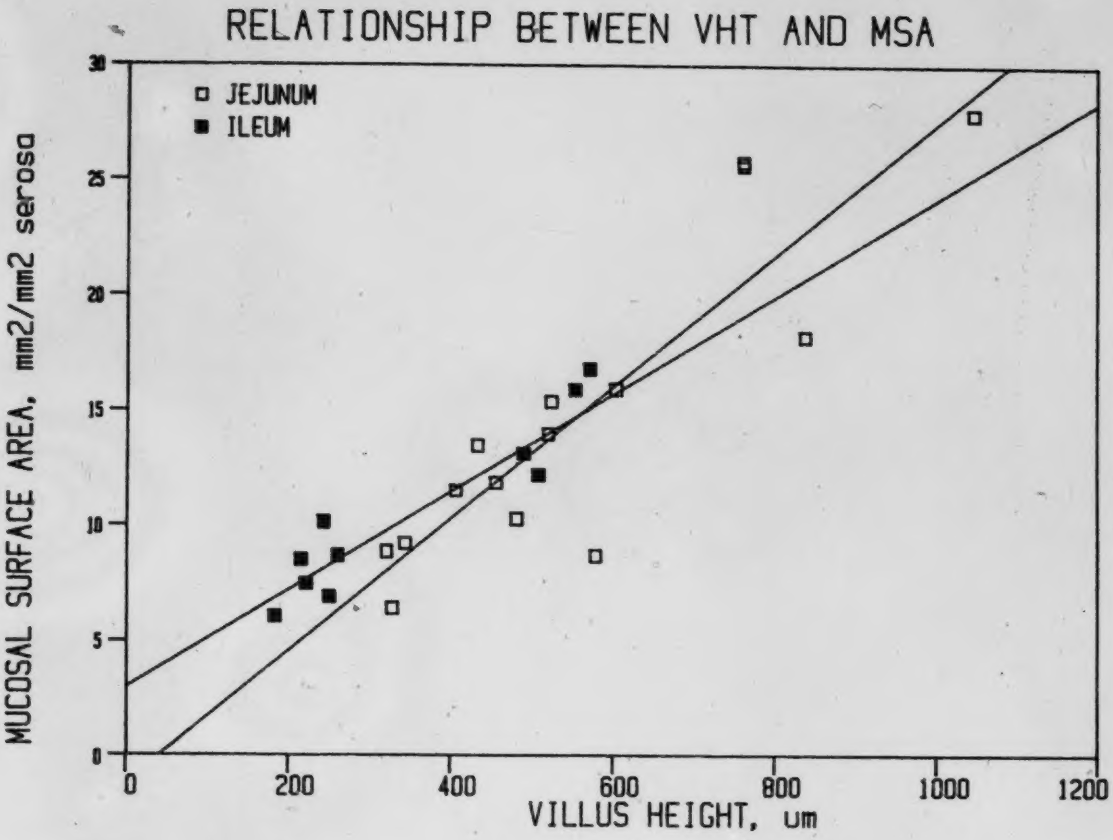

Figure VI-2. Relationship between villus height and mucosal surface area.

(jejunum: r=0.886, p<0.01; ileum: r=0.931, p<0.01)

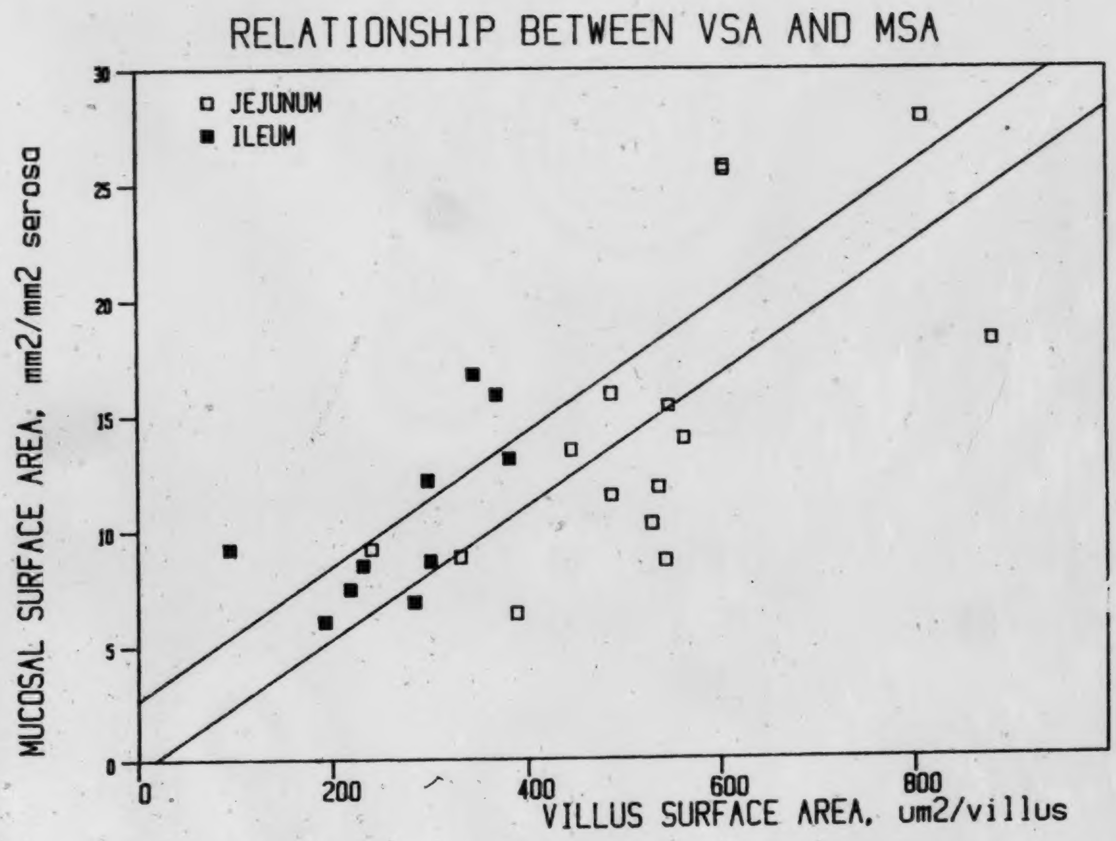

Figure VI-3. Relationship between villus surface area and mucosal surface area.

(jejunum: r= 0.688, p<0.01; ileum: r= 0.625, p<0.01)

TABLE VI -1 ALTERATIONS IN NUTRIENT TRANSPORT, CELL MATURITY, LIPIDS AND PHOSPHOLIPIDS IN FOUR ANIMAL MODELS

	Nutrient Transport		Cell Maturity	Lipids	Phospholipids		
Animal Model	Active	Passive	AP/S	PL/C	Choline	Amine	C/A
Aging	↑	↓	↓	↑	↑-↓	NC-↑	↑-NC
Diabetes	↑	↑	NC	↑	↑	↑	NC
Chronic Ethanol Consumption	↓-NC	↓	↓	↓	↓	NC	↓
External Abdominal Irradiation	↓	↓	↑	↑	↑	↑	NC

TABLE VI-2. MECHANISMS INVOLVED IN CARRIER-MEDIATED TRANSPORT ADAPTATION

Model	Km	Vmax
Aging	+	+
Diabetes	-	+
Ethanol	+	+
Irradiation	+	+

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CHAPTER VII

RECOMMENDATIONS FOR FUTURE RESEARCH

The four animal models studies in this thesis have revealed that BBM adaptation occurs via changes in its phospholipid composition, which in turn is associated with the functional adaptation in nutrient transport. The transport of nutrients can be further investigated with the use of BBM vesicles, and the lipid composition assessed with the use of quantitative methods. The control of the BBM lipid composition can be assessed by studying the rate-limiting enzymes for their incorporation, as well as the turnover of lipids in the BBM.

In order to obtain more conclusive evidence for the relationship between membrane lipid composition and function, several questions must be answered.

1) Is the cholesterol content of the BBM regulated?

I would like to answer the question, "Is the cholesterol content of the brush border membrane regulated"?, as suggested by Brasitus and Schachter (1982). The activity of the rate-limiting enzyme for cholesterol synthesis may be measured in each of the models studied. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) activity can be measured on mucosal homogenates using the assay of Philipp and Shapiro (1979). The systematic incorporation and turnover of cholesterol into the membrane may be measured following subcutaneous injection of [1-^{14}C]-acetate or radiolabelled water. A series of animals would be sacrificed at several time intervals, the tissue collected, membranes purified and counted (Perrodin and Lutton, 1985; Perrodin et al, 1985). Perfusion of the intestine with labelled acetate or water would allow the assessment of the luminal route of incorporation and turnover of cholesterol in the membrane. If the activity of HMG-CoA reductase is changed and the rate of incorporation of radiolabelled cholesterol is altered under conditions where BBM cholesterol content remains stable, then it may be concluded that intestinal brush border membrane cholesterol content is regulated and therefore represents one of the possible control steps in the maintenance of intestinal composition.

It would also be of interest to distinguish between villus and crypt cell HMG-CoA reductase activity. The cholesterol content may vary along the villus so that the "apparent" cholesterol content is unchanged for the villus, yet the tip cell cholesterol is altered. A monoclonal antibody to HMG-CoA reductase would be useful for the localization of the enzyme in cells along the villus. Cells may be isolated along the crypt/villus unit so that the incorporation of cholesterol into the membrane may be assessed. This would be of interest so that it may be determined whether the cholesterol incorporated into crypt cells is maintained for the life of the enterocyte as it migrates up the villus, or whether the cholesterol turns over several times before the cell is sloughed from the villus tip.

2) How is BBM phospholipid content regulated?

The activity of the enzymes of phospholipid synthesis may be measured in each of the animal models studied. CTP:phosphocholine cytidyltransferase is thought to be the rate-limiting enzyme for phosphatidyl choline biosynthesis. CDP-choline: 1,2-diacylglycerol choline phosphotransferase is the enzyme involved in the conversion of CDP-choline to phosphatidyl choline. These enzymes can be measured in mucosal homogenates or microsomal preparations (Pelech and Vance, 1984). The regulation of phosphatidyl choline and phosphatidyl ethanolamine biosynthesis in isolated rat intestinal cells has been studied and demonstrates that the cytidylyltransferases are rate-limiting (O'Doherty, 1980). This study also suggests that phospholipid synthesis is affected by the availability of diacylglycerol as well as its fatty acid composition.

Phospholipid incorporation and membrane turnover may be measured by following injection of [^{32}P]-phosphoric acid and [^{3}H]-glycerol. Animals would be sacrificed at several time intervals, tissue collected, membrane purified, extracted and counted (Lee et al, 1980). If the time-course of the changes in the activity of these enzymes parallels the alterations in BBM radiolabelled phospholipid turnover as well as BBM phospholipid content, then these enzymes could be implicated in the control of BBM composition and function. Cells may be isolated along the crypt-villus gradient to determine whether there are any differences in phospholipid incorporation and turnover in the heterogenous villus cell population of immature and mature functioning transport cells. The relationship between perturbations in these phospholipid-regulating enzymes and HMG-CoA reductase could be established and the signals responsible for the changes in BBM lipids could be established.

3) What is the molecular species composition of individual diacyl phospholipids?

Thin-layer argentation chromatography separates the individual diacyl glycerols according to their degree of unsaturation (Holub, 1978) following hydrolysis of the individual phospholipids with phospholipase C (Holub, 1980). The molecular species composition of individual phospholipids along the crypt-villus unit may be important in the assessment of the relationship between BBM phospholipids with membrane properties and membrane-associated proteins such as alkaline phosphatase and the glucose carrier.

4) What is the fatty acid composition of the individual phospholipids?

The phospholipid fatty acid composition may be assessed following methylation of the fatty acids of individual phospholipids separated by thin-layer chromatography. The fatty acid methyl esters are quantitated by glass capillary gas/liquid-chromatography (Innis and Clandinin, 1981). The phospholipid fatty acid composition will reveal additional information on the properties of the BBM lipid. If the fatty acids are highly polyunsaturated, the membrane will likely be more permeable due to the loose packing of these hydrocarbon chains. A highly saturated fatty acid composition would likely be associated with a more rigid BBM structure with a decreased permeability. Autoradiography of the villus following [^{3}H]-fatty acids given by mouth and intravenously, will determine if there is any heterogeneity in the uptake and turnover of the fatty acids of individual phospholipids. The nature of the phospholipid fatty acids may also be important in determining the activity of carrier proteins and membrane-bound enzymes.

5) Is the fluidity of the BBM altered in the four models studied?

Measurements obtained by the techniques of fluorescence polarization and density scanning calorimetry have been used to assess the fluidity of rat and rabbit brush border membranes (Brasitus et al, 1980; Mutsch et al, 1983; Schachter and Shinitzky, 1977). These techniques could be used to study fluidity of the BBM in each of the animal models, keeping in mind the limitations of these types of measurements. A relationship could be sought between BBM fluidity, lipid composition and BBM transport function. No simple relationship between cell function and membrane fluidity has been described in mammalian cells. Each fluidity technique monitors a different aspect of the lipid bilayer. Membrane proteins appear to be influenced by the level of unsaturation in the membrane bilayer, therefore it has been suggested that perhaps examination of the motional characteristics of the protein (i.e. carrier protein) may be a more direct approach in defining the relationship between lipid composition and membrane function (Stubbs & Smith, 1984). The possible permeability properties of the BBM may be correlated to the fluidity of the BBM. This could be studied most successfully in an in vitro system with liposomes of extracted BBM lipid.

6) How can cell maturity be assessed?

Several BBM antigens have recently been described by Quaroni (1985) which could be used as markers of cellular differentiation. These antigens distinguish the immature crypt and lower villus cells.

Glycoprotein synthesis has also been suggested as a marker of cellular differentiation (Weiser, 1973). The more mature cells have membrane glycoproteins associated with the membrane-bound enzymes (alkaline phosphatase).

Immature cells do not have the full transporting function associated with mature villus cells. A reduction in transport function may be associated with an increased proportion of immature cells along the villus. An increase in transport function may be associated with an increased proportion of mature cells or a decreased proportion of immature cells along the villus. The establishment of cell maturity may be important in the determination of a possible mechanism by which transport function is altered. The BBM lipid composition may also be altered when comparing immature with mature villus cells. It may be possible that the differences observed in lipid composition may be due to differences in cell maturity, which in turn are related to differences in membrane function.

7) How can dynamic cell kinetics be measured?

At this time there is no information available on the dynamic cell kinetics along the villus of the animal models studied. An important mechanism of adaptation may be revealed by the study of crypt cell birth rate, cell turnover time and cell migration rate. The common denominator would be a change in the number of functionally mature cells.

Crypt cell production rate (CCPR) may be assessed via the metaphase arrest technique (Tannock, 1965; Wright and Appleton, 1980) to give an accurate estimation of cell birth rate and turnover time. An increase in cell birth rate with an unaltered or decreased cell turnover time may result in taller villi. If cell turnover time is also increased, villus height would not change but the number of functionally mature cells and therefore uptake might increase. Accordingly, these measurements of cell turnover may be correlated with alterations in villus microdensitometry for leucine, lysine and fatty acids. This would test the hypothesis that an alteration in crypt cell production rate produces a change in the number of functionally mature enterocytes. A decreased cell birth rate with an unaltered or increased cell turnover time would result in shortened villi. In the animal models where alterations in villus morphology have been observed, CCPR may be useful in defining the mechanism of the changes in villus architecture.

Cell migration rate up the villus may be measured following [^{3}H]-thymidine injection. [^{3}H]-thymidine is incorporated into newly synthesized DNA. The labelled cells migrate from the crypt up the villus. Animals are sacrificed at several time intervals, intestinal sections are taken and autoradiography performed.

A measure of the proportion of functional transporting cells can be obtained via autoradiography studies of tissue exposed to a labelled probe such as [^{3}H]-leucine. The functional surface area of the villus may not be the same for all nutrients. Recent studies have illustrated that lysine and leucine are absorbed only in the upper 1/3 of the villus (Cheeseman, unpublished observations). Further studies are necessary to define the functional cells which transport sugars (glucose) and lipids (cholesterol, fatty acids). It is possible that the villus may maintain a constant proportion of transporting cells. In this situation, an increase in villus height would result in an increase in the absolute number of transporting cells. Another possibility is an increased proportion of transporting cells without an increase in villus height. This also would result in an absolute increase in the number of transporting cells. Several possibilities exist whereby the functional surface area could be maintained without a corresponding change in the static measurements of mucosal surface area performed in the animal models studied. It becomes clear that static morphological measurements may not accurately represent the functional surface area of the intestine. This may explain the lack of any relationship between intestinal morphology and transport function observed in the animal models studied as reported in this thesis.

Ornithine decarboxylase (ODC) has been suggested to have an important role in intestinal maturation, mucosal recovery after injury and mucosal hyperplasia (Luk and Baylin, 1981). Measurement of ODC activity in each animal model may reveal another adaptive response. It is of particular interest to distinguish between differentiation and proliferation. It was first thought that ODC was a marker of cell proliferation, but more recently ODC has been thought to be a marker of differentiation and cell maturity (Cassidy, 1985). This concept results from studies which measure thymidine kinase and ODC activity in crypt cells. Thymidine kinase activity was high in the proliferative crypt compartment, whereas ODC activity was absent. ODC activity was found only in the upper villus cells, and correlated well with the activity of mature cell enzymes (sucrase, alkaline phosphatase). ODC was not correlated with DNA synthesis or content.

Measurements of these dynamic cell parameters in each of the animal models would give a greater insight into the dynamic morphological adaptation response of the intestine.

8) Can the heterogeneous cells of the villus be studied?

Cells can be isolated along the crypt-villus gradient and studied for differences in membrane composition and function. Cells can be eluted sequentially as described by Weiser (1973), and Bickel and Munson (1986), or by centrifugal elutriation (Barber et al, 1986). Current studies have measured the enzyme activity, lipid composition and transport function of the entire villus unit. It may be more appropriate to isolate and study the gradient of immature-to-mature cells along the villus. The changes observed in each cell population may be much more dramatic than any of the changes previously reported since there would no longer be any masking by "contaminating" cells from other sites along the villus.

9) How can the effects of specific lipid modifications be assessed?

Recent studies have demonstrated that modifications in dietary fat alter membrane function and lipid composition of kidney, brain, heart and liver (Clark et al, 1983; Foot et al, 1982; Innis and Clandinin, 1981). A study on the effects of feeding rats a high polyunsaturated fatty acid diet or a high saturated fatty acid diet for a period of two weeks did alter intestinal transport function, but without a change in the lipid composition (Thomson et al, 1986). Fatty acid analyses of the intestinal BBM phospholipids were not done at that time, but were performed in a subsequent study and were found to be altered by diet (Keelan et al, 1986). Further studies are required to examine the effects of modifying dietary lipid on BBM composition and function utilizing _in vivo_ and _in vitro_ techniques.

Fetal intestinal organ culture (FIOC) is a useful model to study the influence of dietary (exogenous) lipid on cell maturation, differentiation, and function (Quaroni, 1985). These cells retain their morphological and functional characteristics of the _in vivo_ system. The surface is populated by a single layer of intestinal epithelial cells expressing the most differentiated cell surface markers of cells _in vivo_. Cultured systems and even isolated cells are still too complex to establish conclusive evidence for the relationship between lipid composition and function, especially if the change is produced while the tissue is in the intact animal.

BBM and basolateral membrane (BLM) vesicles may be prepared and the lipid composition modified by incubation with liposomes and a phospholipid exchange protein (Barsukov et al, 1980). It would then be possible to achieve a given change in the membrane lipid composition, which could be assessed by lipid analyses of the vesicles. In vitro uptake studies of glucose, fatty acids, cholesterol and phospholipids could be performed with control (unmodified) and modified membrane vesicles to determine the direction and magnitude of the resulting changes in permeability at both entry and exit sites of the enterocyte. Membrane vesicles may be modified in vivo by dietary manipulation in the intact animal or artificially in vitro by incubation of membrane vesicles with liposomes containing different ratios of lipids. These studies would allow the examination of the effect of membrane lipid changes on active and passive nutrient uptake. Intestinal permeability changes produced by dietary manipulation can also be assessed by in vitro uptake studies using flat sheets of intestine or by in vivo perfusion studies.

10) How can dietary manipulation be used to study the relationship between membrane lipid comosition and function?

Diet may be a useful perturbation by which the mechanism of changes in lipid composition and function may be explored. The objective will be to define and identify the minimum change in membrane lipid composition required to produce a change in membrane function. Dietary cholesterol, phospholipid and fatty acids could be modified, and the membranes isolated for studies of transport function and lipid composition. As mentioned previously, two diets differing in the ratio of polyunsaturated/saturated fatty acid diets produced a change in the fatty acid composition of the BBM phospholipids (Keelan et al, 1986). Continued studies are required to assess the permeability properties of

BBM vesicles modified by diet. In addition, a time course study of the lipid synthesizing enzyme changes in response to a diet stimulus would be useful in understanding the mechanism behind the membrane function changes.

The phospholipid fatty acids may be the key to the relationship between membrane lipid composition and function. If it is possible to establish the effects of altering the lipid composition on membrane function with particular fatty acids supplied exogenously by diet (in vivo manipulation), liposomes or culture medium (in vitro manipulation), it may be possible to predict functional changes produced by diet, and possibly correct abnormal membrane function in health and disease.

To continue the study of the regulation of lipid incorporation into membranes, fatty acid metabolism will need to be examined in addition to the metabolism of cholesterol and phospholipids. Desaturase and elongation enzymes are of interest as they produce the long polyunsaturated fatty acids. These enzymes could be measured in mucosal homogenates and microsomal preparations (Garda and Brenner, 1985).

Studies on the regulation of membrane lipid composition, physical properties of the membrane, cell maturity, dynamic cell kinetics, and the manipulation of membrane lipid composition by exogenous (dietary) lipid will contribute to a better understanding of the basic processes in intestinal adaptation. On a wider horizon it would be possible to establish the principles of membrane manipulation which modify membrane function in other tissues. The relationship between in vivo and in vitro uptake studies would establish a continuation of the interest in intestinal uptake in the clinical control of diseases such as diabetes.

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APPENDIX 1

ISOLATION AND PURIFICATION OF THE BRUSH BORDER MEMBRANE

The isolation and purification methods used to establish the marker enzymes and lipid content of the brush border membrane (BBM) were based on the work of Yakymyshyn et al (1982). For the studies using the rabbit model, 30 cm each of proximal jejunum and distal ileum were removed from each rabbit and placed into ice-cold saline. For the studies using the rat model, 40 cm of intestine was obtained from each of the two sites. Each segment was irrigated three times with ice-cold saline and placed on a pre-chilled glass plate. The segments were opened longitudinally along the mesenteric border and the mesentery was removed. The mucosal surface was blotted with lint-free tissue to remove excess moisture. The mucosal surface was removed by gently scraping with a microscope slide, then placed into pre-weighed glass vials containing 5.0 ml of 50 mM mannitol-2 mM Tris buffer pH 7.1, and frozen at -80°C for later purification of the BBM. Sections, 1 cm in length, were taken before and after mucosal scraping and placed in Bouin's solution for preparation of paraffin sections for light microscopy to determine the depth of scraping as well as villus morphology (Appendix 4).

The isolation and purification of the brush border membrane is summarized in Figure A1-1. Mucosal scrapings were transferred from the glass vial to a 250 ml polypropylene bottle. The scrapings were then homogenized in a total volume of 115 ml mannitol-Tris buffer using a Brinkman Polytron® at setting "8" for 15 seconds. The homogenate was filtered through gauze, a metal sieve, and a Pharmacia 40 M millipore filter to remove mucus and debris. A 1M solution of $CaCl_2$ was added to the homogenate to obtain a final concentration of 10 mM $CaCl_2$ (1.0 ml 1 M $CaCl_2$ per 100 ml homogenate) and stirred gently for 10 minutes on ice with a magnetic stirrer to precipitate the subcellular components. The homogenate was centrifuged at 3000 x g for 15 min at 4°C to pellet the subcellular components; the pellet P_1 was discarded. The supernatant S_1 was taken off and centrifuged at 43700 x g for 20 min at 4°C. The supernatant S_2 was discarded. The pellet P_2 was resuspended in 30 ml of 50 mM mannitol-2mM Tris-5 mM EGTA buffer pH 7.1 and centrifuged at 43700 x g for 20 min at 4°C. The supernatant S_3 was discarded. The pellet P_3 was resuspended in 2.0 ml deionized water and sonicated (Ultrasonics cell disrupter sonicator W-375). The sonicated P_3 preparation was layered onto 25 ml of 40% Percoll® (Pharmacia) and centrifuged at 43700 x g for 20 minutes at 4°C. The upper fraction (p=1.019-1.055 g/ml) contained the BBM. Contaminating core material remained in the lower fraction. The Percoll® was removed from the BBM by centrifuging at 115000 x g for 45 min at 15°C. The final BBM preparation was resuspended in the supernatant, then aliquotted for immediate lipid extraction (Appendix 3). Additional aliquots for marker and lipid analyses were stored at -80°C.

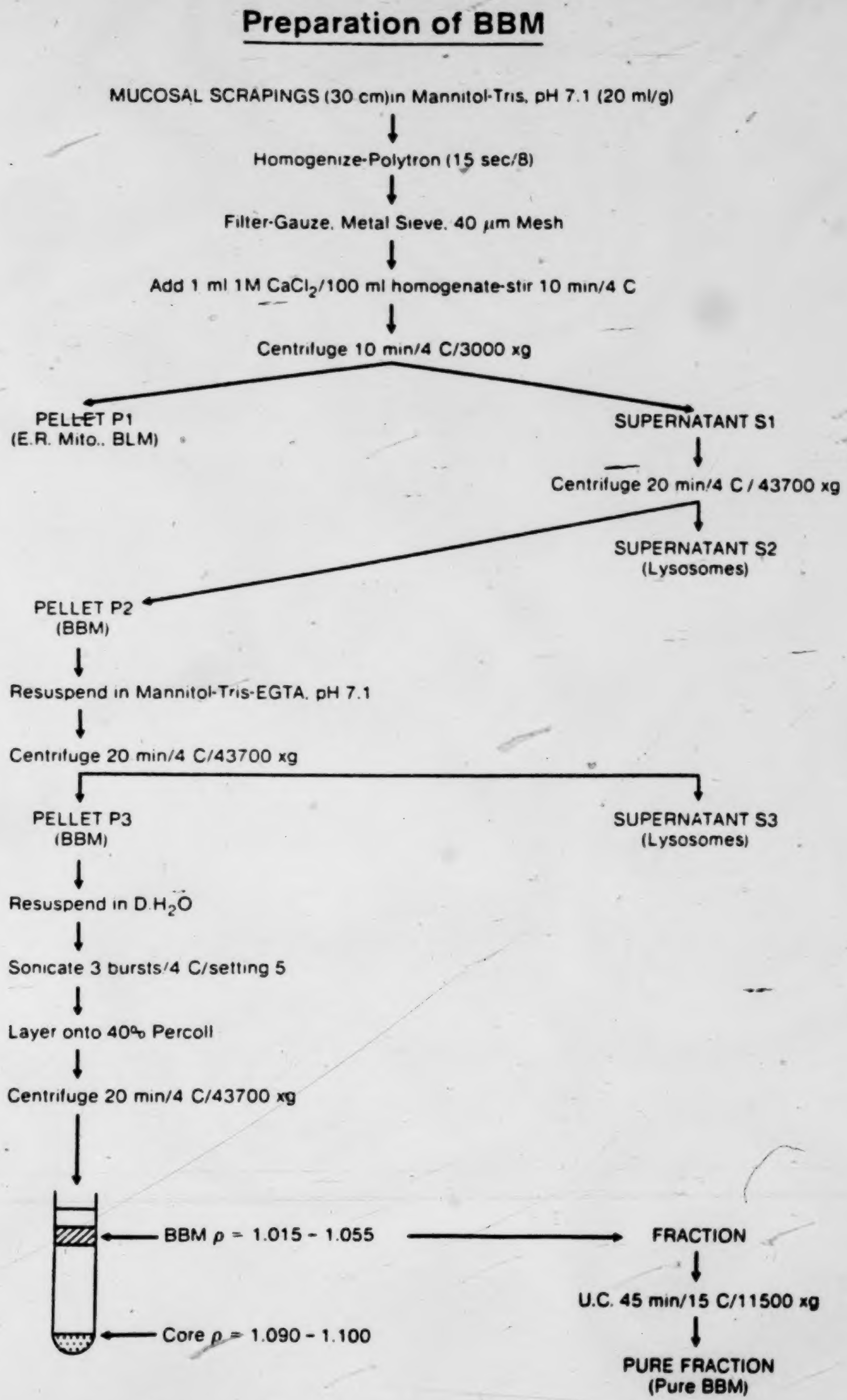
Preparation of BBM
MUCOSAL SCRAPINGS (30 cm)in Mannitol-Tris, pH 7.1 (20 ml/g)
Homogenize-Polytron (15 sec/8)
Filter-Gauze, Metal Sieve, 40 μm Mesh
Add 1 ml 1M $CaCl_2$/100 ml homogenate-stir 10 min/4 C
Centrifuge 10 min/4 C/3000 xg
PELLET P1
(E.R. Mito., BLM)
SUPERNATANT S1
Centrifuge 20 min/4 C / 43700 xg
SUPERNATANT S2
(Lysosomes)
PELLET P2
(BBM)
Resuspend in Mannitol-Tris-EGTA, pH 7.1
Centrifuge 20 min/4 C/43700 xg
PELLET P3
(BBM)
SUPERNATANT S3
(Lysosomes)
Resuspend in D.H_2O
Sonicate 3 bursts/4 C/setting 5
Layer onto 40% Percoll
Centrifuge 20 min/4 C/43700 xg
BBM ρ = 1.015 - 1.055
Core ρ = 1.090 - 1.100
FRACTION
U.C. 45 min/15 C/11500 xg
PURE FRACTION
(Pure BBM)

Figure A1-1. Isolation and purification of brush border membranes.

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APPENDIX 2

ENZYME MARKER ANALYSES

TOTAL PROTEIN was determined using the Hartree modification of the method of Lowry (Hartree, 1972; Lowry et al, 1951). BBM protein initially interacts with Cu^{++} in alkali for 10 minutes at 45°C. The Cu-protein complex and the tyrosine and tryptophan of the protein then reduce phosphotungstic and phosphomolybdic acids (Folin-Ciocalteu reagent) to molybdenum blue and tungsten blue while standing at 20°C for 30 minutes. The absorbance was measured at 740 nm.

$$\text{BBM protein} + \text{Cu}^{++} \xrightarrow[45^\circ\text{C}]{} \text{Cu-protein}$$

$$\begin{array}{l}\text{Cu-protein}\\ \text{tyrosine}\\ \text{tryptophan}\end{array} + \text{phosphotungstic acid} + \text{phosphomolybdic acid} \xrightarrow[20^\circ\text{C}]{} \underset{A_{740}}{\text{tungsten } \underline{\text{blue}} + \text{molybdenum } \underline{\text{blue}}}$$

The standard curve ranged from 30-150 μg protein (bovine albumin standard). To ensure quality control, a serum control and a BBM control were assayed with every run. All enzyme marker activity was expressed as specific activity, U/g protein, which is the amount of enzyme required to utilize 1 μmole of substrate per minute at 37°C per gram protein.

MARKERS OF MEMBRANE PURITY

ALKALINE PHOSPHATASE activity was assayed by the method of Bowers et al (1967) which utilized the enzymatic hydrolysis of p-nitrophenylphosphate

(PNPP) to p-nitrophenol (PNP) by alkaline phosphatase (AP). The BBM preparation was incubated with 0.625M 2-amino-2-methyl-1-propanol (AMP) -2mM magnesium chloride buffer, pH 10.25 at 37°C. The magnesium chloride acted as an accelerator for AP. The substrate, 50 mM PNPP, was then added as the starting reagent. The hydrolysis product, PNP, is highly coloured (yellow) under alkaline conditions. The increasing yellow colour was measured at 410 nm every 30 seconds for 3 minutes.

$$\text{PNPP} \xrightarrow[\text{AMP-MgCl}_2\text{; 37°C, pH 10.25}]{\text{BBM-AP}} \underset{\substack{\text{(yellow)} \\ A_{410}}}{\underline{\text{PNP}}} + H_3PO_4$$

This assay utilized rate reaction kinetics, therefore linearity (with respect to the amount of membrane protein added to the assay system) was visible and demonstrated by every sample assayed. To ensure quality control, a serum control and a BBM control were assayed at the beginning and end of every run.

SUCRASE activity was assayed by the method of Dahlqvist (1964). The BBM preparation is incubated with 0.1 M maleate - 56 mM sucrose buffer, pH 6.0 for 30 minutes at 37°C. Sucrose is broken down into glucose and fructose by the BBM sucrase. The reaction was stopped by the addition of deionized H_2O and placing assay tubes in the water bath at 100°C for 2 minutes. The glucose is measured by the glucose-specific hexokinase reaction whereby glucose in the presence of ATP and magnesium forms glucose-6-phosphate (G-6-P). Glucose-6-phosphate dehydrogenase (G-6-PDH) in the presence of NAD converts G-6-P to 6-phosphogluconate and NADH. The amount of NADH produced is proportional to the glucose formed by the BBM sucrase activity. The absorbance of NADH is measured at 340 nm using the Worthington Instrument Application for the ABA-100 bichromatic auto-analyzer (Cat. Nos. 7921, 7922).

$$\text{Sucrose} \xrightarrow[\substack{\text{maleate, }37^\circ\text{C} \\ \text{pH }6.0}]{\text{BBM-sucrase}} \text{glucose} + \text{fructose} \xrightarrow[100^\circ\text{C}]{H_2O} \text{(stop reaction)}$$

$$\text{Glucose} + \text{ATP} \xrightarrow[Mg^{2+},\ 37^\circ\text{C}]{} \text{Glucose-6-phosphate (G-6-P)}$$

$$\text{G-6-P} + \text{NAD} \xrightarrow[37^\circ\text{C}]{\text{G-6-PDH}} \text{6-phosphogluconate} + \frac{\text{NADH}}{A_{340}}$$

This assay utilized endpoint kinetics, therefore the assays were performed with the addition of different amounts of BBM protein to demonstrate the linearity of the reaction. The glucose standard curve ranged from 25-200 mg/dl. To ensure quality control, a serum control and a BBM control were included at the beginning and end of every run.

MARKERS OF MEMBRANE CONTAMINATION

Na+K+-ATPase activity was assayed according to the method of Scharschmidt and coworkers (1979), and was used as a marker of basolateral membrane (BLM) contamination. Membrane-associated Mg^{2+} activated Na+K+-ATPase activity was determined by a coupled enzyme assay in which the formation of ADP by ATPase in the presence and absence of ouabain is coupled to NADH oxidation with intermediate enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH) and intermediate substrate phosphoenolpyruvate (PEP) present in excess.

A stock reaction buffer (Scharschmidt et al, 1974) containing β-NADH and PEP with (B) or without (A) ouabain was incubated 3 minutes at 37°C with $MgCl_2$-ATP solution. The reaction begins with the addition of LDH/PK and the BBM preparation. NADH oxidation was monitored as decreasing absorbance at 340 nm every 60 seconds for 6 minutes. The ouabain insensitive ATPase activity (B) was subtracted from the total ATPase activity (A) to obtain the ouabain sensitive fraction, which represents the activity of the Na+-K+-ATPase.

$$\mathrm{ATP} \xrightarrow[\substack{\mathrm{MgCl_2,\ 37^\circ C} \\ (^{+}/_{-}\ \text{ouabain})}]{\text{BLM ATPase}} \mathrm{ADP} + \mathrm{Pi}$$

$$\mathrm{ADP} + \mathrm{PEP} \xrightarrow[37^\circ\mathrm{C}]{\mathrm{PK}} \text{pyruvate} + \mathrm{ATP}$$

$$\text{Pyruvate} + \underset{A_{340}}{\underline{\beta\text{-NADH}}} + \mathrm{H}^{+} \xrightarrow[37^\circ\mathrm{C}]{\mathrm{LDH}} \text{lactate} + \underline{\mathrm{NAD}^{+}}$$

This assay utilized rate reaction kinetics, therefore linearity (with respect to the amount of membrane protein added to the assay system) was visible and demonstrated by every sample assayed.

β-GLUCURONIDASE activity was assayed according to the method of Glaser and Sly (1973), and was used as a marker of lysosomal membrane contamination. The BBM preparation was incubated with 0.2 M acetate buffer, pH 4.0 and methylumbelliferone (MUM)-β-D-glucuronide substrate for 60 minutes at 37°C. The reaction was terminated by the addition of 0.25 M glycine-carbonate buffer, pH 9.9. β-glucuronidase converts the non-fluorescent MUM-β-D-glucuronide to MUM which is highly fluorescent at an alkaline pH. The fluorescence was measured at the excitation/emission spectra of 360 nm/450 nm.

$$\underset{\text{(non-fluorescent)}}{\text{MUM-}\beta\text{-D-glucuronide}} \xrightarrow[\text{acetate, 4.0, 37°C}]{\text{lysosomal }\beta\text{-glucuronidase}} \text{MUM}$$

$$\text{MUM} \xrightarrow[\text{glycine-carbonate buffer, pH 9.9, 20°C}]{} \text{MUM} \qquad \underset{F_{360/450}}{\text{(highly fluorescent)}}$$

This assay utilized endpoint kinetics, therefore the assays were performed with the addition of different amounts of protein to demonstrate the linearity of the reaction. The methylumbelliferone standard curve ranged from 0.5 - 5.0 nmoles. To ensure quality control, an S_2 sample was included in every run.

DNA content was assayed according to Giles and Myers (1965) modification of the method of Burton (1956), and was used as a marker of nuclear contamination. The assay is a modification of the classical Dische reaction of DNA with diphenylamine to produce a blue color. The BBM preparation is incubated with 20% perchloric acid, 4% diphenylamine in glacial acetic acid and 0.16% acetaldehyde for 18 hours at 20°C. The assay tubes are then centrifuged for 10 minutes at 1300 x g. The absorbance of the supernatant was measured at 595 nm.

The DNA standard curve ranged from 5-50 µg. To ensure quality control, a P_1 sample was included in every run.

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APPENDIX 3

LIPID ANALYSES

LIPID EXTRACTION

Approximately one-half of each BBM preparation was immediately extracted by a modification of the method of Folch (Bowyer and King, 1977; Folch et al, 1957) using sequential addition of methanol and chloroform (1:2), followed by acidification of the water wash using dilute sulfuric acid (0.01M). To ensure quality control, a blank and recovery were included in every run. Aliquots from the lower chloroform layer were removed for the determination of total phospholipid, cholesterol (total and free), and thin-layer chromatography. Aliquots were evaporated to dryness under nitrogen at 60°C. The dried extracts were stored at -80°C prior to analysis.

TOTAL FREE FATTY ACID content was assayed using the NEFA-Kit (as supplied by Nippon Shoji Kaisha, Ltd.), which utilizes the method of Mizuno et al (1980) and Okabe et al (1980). Acyl CoA synthetase (ACS) in the prsence of ATP and coenzyme A (CoA) converts free fatty acid to acyl CoA, AMP and pyrophosphate (PPi). Acyl CoA is then oxidized by acyl CoA oxidase (ACO) to 2,3-trans-enoyl-CoA and peroxide. N-ethylmaleimide (NEM) is added to remove any interferences due to the presence of excess CoA). The peroxide generated was determined by oxidative coupling of 4-amino-antipyrine (4-AAP) and N-ethyl-N-(2-hydroxy-3-sulfapropyl)-m-toluidine sodium salt (TOOS) by peroxidase (POD). The absorbance of the resultant purple-red quinione proportional to the amount of free fatty acid present. The fresh BBM preparation was incubated with reagant A (ATP, CoA, acyl CoA synthetase, Mg^{2+}) for 10 minutes at 37°C. Reagent B (NEM, acyl CoA oxidase, 4-AAP, TOOS, POD) was added to the sample, then incubated for 5 minutes at 37°C. The absorbance of the samples was then measured at 500 nm.

$$\underset{\text{(BBM Sample)}}{\text{RCOOH}} + \text{ATP} + \text{CoA} \xrightarrow[\text{Mg}^{2+},\ 37°\text{C}]{\text{ACS}} \text{Acyl CoA} + \text{AMP} + \text{PPi}$$

$$(\text{CoA} + \text{NEM} \xrightarrow[37°\text{C}]{} \text{CoA} - \text{NEM})$$

$$\text{Acyl CoA} + O_2 \xrightarrow[37°\text{C}]{\text{ACO}} \text{2,3-}\underline{\text{trans}}\text{-enoyl-CoA} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{TOOS} \xrightarrow[37°\text{C}]{\text{POD}} \text{purple-red } \underset{A_{550}}{\underline{\text{quinone}}} + 4H_2O$$

The oleic acid standard graph ranged from 10-30 nmoles. To ensure quality control, a serum sample was included with every run.

TOTAL BILE ACID content was assayed fluorimetrically according to the method of Mashige et al (1976) and Osuga et al (1977). 3 α-hydroxy bile acids were converted to their 3-oxo-derivatives by 3 α- hydroxy-steroid dehydrogenase (3 α-HSD) with the concomitant reduction of NAD to NADH. NAD-diaphorase transferred the hydrogen of the generated NADH to resazurin, to form the fluorophore, resorfin. The fluorescence of resorfin was proportional to the amount of bile acid present. An aliquot of the BBM preparation was incubated in 0.10 M tris buffer, pH 9.5 and reagent A (3 α-HSD, NAD-diaphorase, resazurin) for one hour at 20°C. A second BBM aliquot was incubated in tris buffer and reagent B (NAD-diaphorase, resazurin). The fluorescence of the samples was measured at the excitation-emission wavelength of 560nm/580 nm. The net fluorescence of sample in reagent A-reagent B was equal to the fluorescence due to bile acid in the sample.

$$\underset{\text{(BBM sample)}}{3\ \alpha\text{-hydroxy bile acids}} + \text{NAD} \xrightarrow[\text{tris buffer, pH 9.5, 20°C}]{3\ \alpha\text{-HSD}} \text{3-oxo-derivatives} + \text{NADH} + \text{H}^+$$

$$\text{H}^+ + \text{resazurin} \xrightarrow[\text{tris buffer pH 9.5, 20°C}]{\text{NAD-diaphorase}} \frac{\text{resorfin}}{F_{560/580}}$$

The chenodeoxycholic acid standard curve was linear and ranged from 0.26 to 5.10 nmoles. To ensure quality control, a recovery was performed with every run.

TOTAL PHOSPHOLIPID content was assayed from BBM lipid extracts digested with 2.5 M sulfuric acid and 30% peroxide according to the method described by Sunderman and Sunderman (1960). The liberated phosphorus was then reacted with 2.5% ammonium molybdate. The phosphomolybdate formed was reduced by 2.5% 1,2,4-amino-naphthol-sulfuric acid (1,2,4-ANSA) in 15% sodium sulfite to form a blue colour, which is proportional to the amount of phosphorus present. The absorption maximum of this blue color was measured at 675 nm.

$$\underset{\text{(Folch extract)}}{\text{BBM phospholipid}} \xrightarrow[\text{100°C}]{\text{2.5M } H_2SO_4\text{, 30\% } H_2O_2} \text{Pi}$$

$$\text{Pi} + \text{ammonium molybdate} \xrightarrow[\text{20°C}]{} \text{phosphomolybdate}$$

$$\text{phosphomolybdate} + \text{1,2,4-ANSA} \xrightarrow[\text{20°C}]{NaHSO_3} \frac{\text{blue color}}{A_{675}}$$

The phosphorus measured was converted to phospholipid by a multiplication factor of 25, based on an average 25 mg phospholipid per mg of phosphorus. The phosphorus standard curve was linear and ranged from 0.01 to 0.04 mg. To ensure quality control, a Folch recovery sample and blank were assayed with each run.

TOTAL CHOLESTEROL content was determined on a dried BBM lipid extract reconstituted with 2-propanol as described by Allain et al (1974) and Morin (1976). Enzymatic determination of total cholesterol required the initial hydrolysis of cholesterol esters by cholesterol esterase, followed by the conversion of cholesterol to cholest-4-en-3-one and peroxide by cholesterol oxidase. The generated peroxide coupled oxidatively with 4-aminophenazone and phenol in the presence of peroxidase (POD) to yield a quinoneamine dye with an absorption maximum at 500 nm.

$$\underset{\text{(Folch extract)}}{\text{BBM cholesterol esters}} \xrightarrow[37^{\circ}\text{C}]{\text{cholesterol esterase}} \text{free cholesterol + fatty acids}$$

$$\text{BBM free cholesterol} + O_2 \xrightarrow[37^{\circ}\text{C}]{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow[37^{\circ}\text{C}]{\text{POD}} \underset{A_{500}}{\underline{\text{quinoneamine dye}}} + 2H_2O$$

FREE CHOLESTEROL content was determined by a similar procedure as described for total cholesterol except that cholesterol esterase was omitted from the reagent mixture.

CHOLESTEROL ESTER content was indirectly determined by subtraction of the free cholesterol from the total cholesterol content.

The free cholesterol standard curve was linear and ranged from 50 to 200 mg/dl. To ensure quality control, a Folch blank, a Folch recovery sample and a serum control was assayed with every run.

RELATIVE LIPID COMPOSITION was estimated by high-performance-thin-layer chromatography (HPTLC) of dried BBM lipid extracts reconstituted in chloroform: methanol (2:1) spotted onto 10 x 10 cm HPTLC plates silica gel 60 (Merck). The plates were developed for 12 minutes in a solvent system containing petroleum ether: diethyl ether: glacial acetic acid (42:9:0.5) (Freeman and West, 1966). Plates were allowed to air dry and then sprayed with Goswami Spray reagent (Goswami and Frey, 1974), followed by a development in an 80°C oven. Semi-quantitative determination of lipids was obtained by scanning densitometry (Helena R + D densitometer). Lipid standard containing lecithin, cholesterol, oleic acid, cholesterol myristate and tristearin were included on every plate. The lipid class separtion is illustrated in Figure A3-1.

RELATIVE PHOSPHOLIPID COMPOSITION was estimated by HPTLC of dried extracts reconstituted in chloroform: methanol (2:1) spotted onto 10 x 10 cm HPTLC plates silica gel 60 (Merck). Phospholipids were separated in a solvent system containing chloroform:methanol:2-propanol:triethylamine:water (15:4.5:12.5:6.5:3.5) (Vitiello and Zanetta, 1978; Touchstone et al, 1979). Plates were air-dried, then immersed in 3% cupric acetate in 8% phosphoric acid, blotted carefully and charred at 180°C. Semi-quantitative determination of phospholipids was obtained by scanning densitometry (Helena R + D densitometer).

Phospholipid standards containing lysolecithin, lecithin, sphingomyelin, phosphatidic acid, phoshatidyl serine, phosphatidyl inositol, phosphatidyl ethanolamine, lysophosphatidyl ethanolamine, and cerebrosides were included on every plate. The phospholipid separation is illustrated in Figure A3-2.

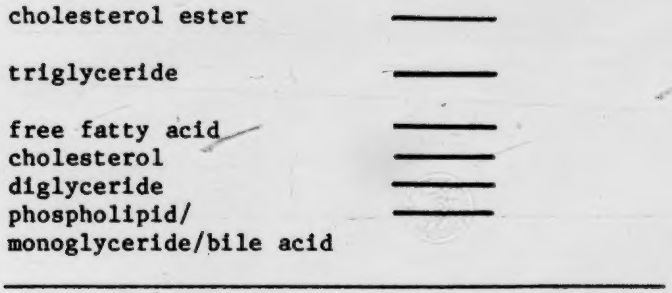

Figure A3-1. Lipid class separation by HPTLC

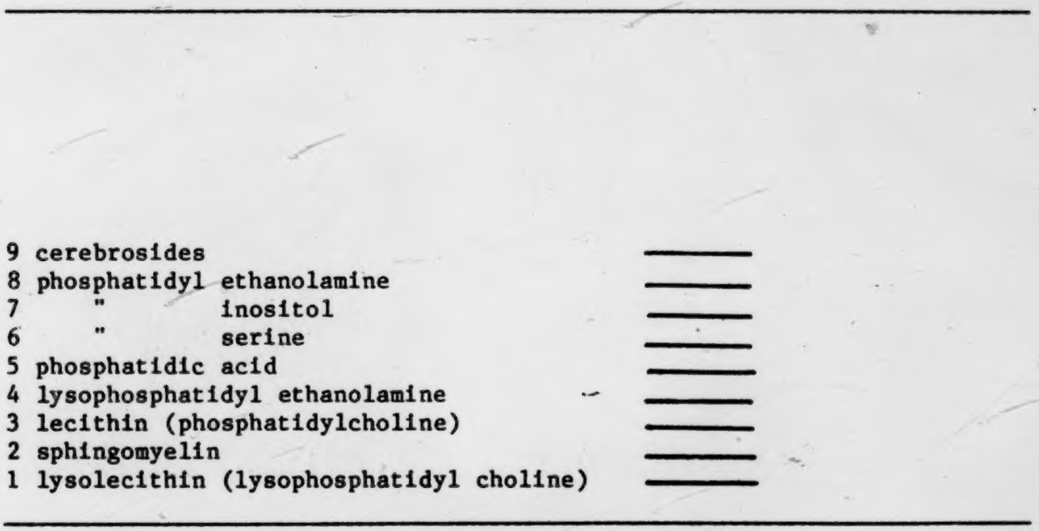
9 cerebrosides
8 phosphatidyl ethanolamine
7 " inositol
6 " serine
5 phosphatidic acid
4 lysophosphatidyl ethanolamine
3 lecithin (phosphatidylcholine)
2 sphingomyelin
1 lysolecithin (lysophosphatidyl choline)

Figure A3-2. Phospholipid separation by HPTLC

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APPENDIX 4

INTESTINAL VILLUS MORPHOLOGY

Over the years intestinal villus morphology has been described in many different ways. Initially, simple measurements of villus height were used as estimates of villus morphology. Several workers have attempted to assess the actual mucosal surface area (Kapadia and Baker, 1976; Boyne et al, 1966; Penzes and Skala, 1977; Clarke, 1972). Some workers included a measure of intestinal circumference in their calculations (Boyne et al, 1966; Penzes and Skala, 1977). Clarke (1972) also included the number of villi per mm^2 serosa in his calculation of mucosal surface area. None of these workers, however, provided a true estimation of villus architecture since they all assumed a constant villus shape.

Ecknauer and coworkers (1982) established a model for the estimation of villus architecture. Measurements of villus height, villus width at half height, villus thickness at half height, and villus bottom width were used to provide a realistic estimate of the surface area of the villus (Figure A4-1). Villus surface area was then calculated as follows:

$$\text{Villus surface area} = (2xMxH)+(2xM-A)xD+(2xDx(((A-M^2)+(H)^2))^{0.5} \text{ x } 1000 \ (\mu m^2/\text{villus})$$

where H = villus height, M=villus width at 1/2 height, A = villus bottom width, D = villus thickness at 1/2 height.

In order to perform the above measurements, tissue sections 1 cm x 1 cm were obtained from each animal, fixed in Bouin's solution, dehydrated and embedded in paraffin wax. Sections were cut at a thickness of 5 μm for light microscopy, mounted on glass slides and stained with hematoxylin and eosin using standard techniques.

Slides were then projected via the use of a Scopicon (projecting microscope). The projected image was calibrated with the use of a micrometer scale, such that the projected image was magnified 1000 times. The measurements of villus height, villus width at 1/2 height, villus bottom width and crypt depth were obtained from vertical tissue sections. Measurement of villus thickness was obtained from transverse tissue sections.

The number of cells per villus were counted on vertical tissue sections. Estimations of villus cell size were made based on the number of cells per villus and villus height.

To get an estimate of villus density, the number of villi per mm were measured in longitudinal and horizontal cross-sections, then multiplied together to obtain the number of villi per mm^2 serosa. When this villus density is multiplied by the villus surface area, the result is the mucosal surface area expressed as mm^2/mm^2 serosa.

$$\underset{(mm^2/mm^2\ \text{serosa})}{\text{Mucosal surface area}} = \text{no. of villi}/mm^2\ \text{serosa} \times \underset{(mm^2/\text{villus})}{\text{villus surface area}}$$

At least 10 villi were assessed per section.

Microvillus morphology has not been included in this calculation of mucosal surface area. Microvillus height and width varies considerably along the villus - they are tallest and narrowest on the upper villus tip cells. Generally microvilli increase the surface area approximately 25 times. This factor could have been included in the calculation.

In some of the studies performed, microvillus morphology was assessed (Chapter III and V). Additional sections were fixed in 4% glutaraldehyde in phosphate buffered saline (pH 7.0), dehydrated and embedded in epoxy resin. The sections were cut less than 0.1 μm thick and a negative stain applied for visualization by conventional transmission electron microscopy. Microvillus height and width were measured only from the cells of the villus tip.

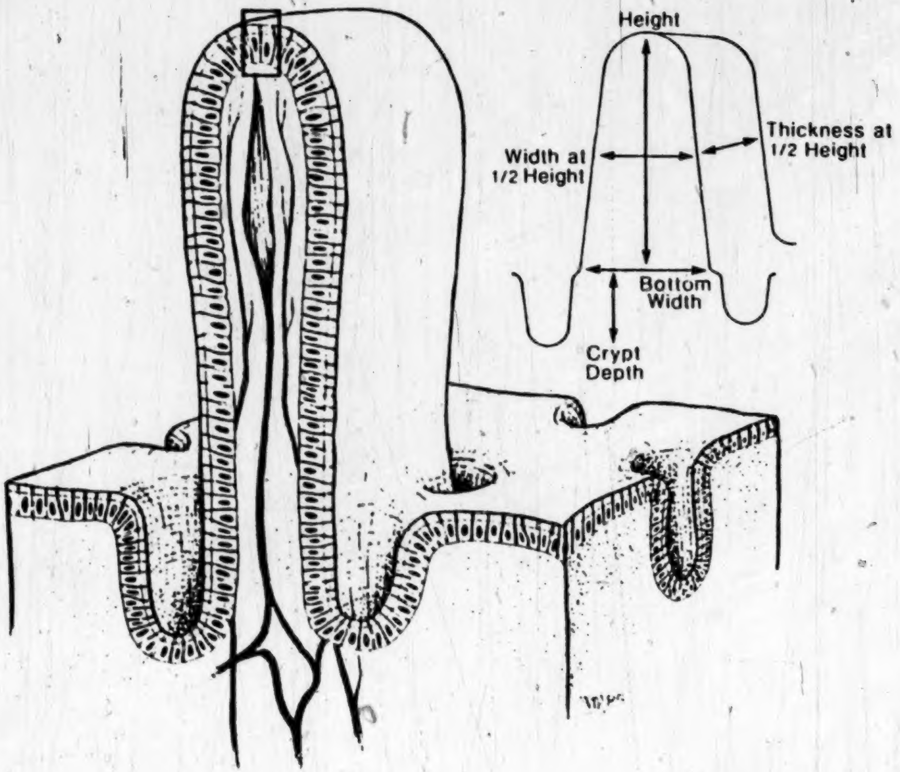

Figure A4-1. Villus measurements for the calculation of surface area.

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A. Manuscripts

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D. Presentations

Intestinal morphology, marker enzymes, and lipid content of brush border membranes from rabbit jejunum and ileum: effect of aging. Presented to American Gastroenterological Association. New Orleans, Louisiana, U.S.A. May 1984.

Correlation between changes in morphology and brush border membrane lipid composition with alterations in transport in aging rabbit intestine. Presented to Canadian Biochemical Society. Banff, Alberta, Canada. May 1984.

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approved by the Review Committee - February 6, 1985 - Edmonton, Alberta Canada.